A CAUSE OF THE THROMBOCYTOPENIA AND LEUKOPENIA THAT OCCUR IN DOGS DURING DEEP HYPOTHERMIA

By TULIO J. VILLALOBOS,[1] EDWARD ADELSON, PHILIP A. RILEY, JR.,[2] AND WILLIAM H. CROSBY WITH THE TECHNICAL ASSISTANCE OF HAROLD GLAUCKE

(From the Department of Hematology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.)

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A previous report from this laboratory (1) presented the results of hematologic studies in nine dogs cooled below 20° C. During induction of hypothermia the animals showed a marked rise in red cell count, hemoglobin, and hematocrit, together with a nearly complete disappearance of white cells and platelets. On rewarming, the values all returned to normal. A number of studies have been carried out to learn more about the mechanism of these changes. The present paper describes some of the studies dealing especially with the changes in platelets and white cells.

MATERIALS AND METHODS

The experiments were performed on a group of 22 adult mongrel dogs. Hypothermia was induced by a method of surface cooling previously reported (2). Each dog was anesthetized with intravenous thiopental. An endotracheal catheter was inserted and connected to an automatic respirator utilizing room air. Thermocouples in the rectum and esophagus permitted continuous temperature recordings by a Brown potentiometer. A continuous three limb lead electrocardiogram and aortic blood pressure were also recorded. The animal was placed in an ice bath at 4° C. and cooled until ventricular fibrillation occurred or the rectal temperature reached 17° C. The heart was defibrillated electrically. Each experiment, from the onset of hypothermia until rewarming had raised the body temperature to approximately 30° C., occupied approximately five hours. Platelet counts were carried out by the method of Brecher and Cronkite (3) using phase microscopy. Two pipettes were filled and two chambers for each pipette were counted and the average was taken as the result for each determination. By this technique, the normal range for platelet counts in dogs is 200,000 to 350,000 per cu. mm. Leukocyte counts were carried out by the standard hemocytometer technique using 1:20 dilution. Both sides of two hemocytometer chambers were counted. Hemoglobin concentration was measured by the cyanmethemoglobin method (4). Hematocrit was measured in capillary tubes after high-speed centrifugation (5).

Protocol 1: Bone marrow aspirations and biopsies were carried out from the ribs and tibias of three dogs before, during, and after hypothermia.

Protocol 2: Studies with radioactive platelets were carried out in dogs using a donor (Dog A) and a recipient (Dog B). Two mc. of P^{32} ($NaHP^{32}O_4$, Abbott) was injected into Dog A. One week later, Dog A was bled by cardiac puncture, and 500 ml. of blood was drawn into a plastic bag containing disodium versinate solution. Simultaneously 500 ml. of blood was taken from Dog B. Dog B was then transfused immediately with the blood from Dog A containing radioactive platelets. One-half hour later, using siliconized equipment and a two syringe technique, 15 ml. of blood was taken from Dog B. Platelets and plasma were separated from red cells by differential centrifugation and the platelets were counted in a hemocytometer chamber using a phase-contrast microscope. The platelet suspension was then centrifuged to throw down the platelets. The platelets were washed three times with saline and transferred to an aluminum planchet and the P^{32} radioactivity was measured in a Geiger-Müller counter with a window thickness of 2.2 mg. per cm.^{2} (Tracerlab). Leukocyte, erythrocyte, and platelet counts, using standard methods, were also performed on the whole blood. Dog B, anesthetized with Pentothal®, was then placed in ice until his body temperature fell to 17° C. and his platelet count fell from 204,000 to 58,000. He was then rewarmed. When his platelet count had risen, a second 15 ml. sample of blood was drawn and the procedure of counting described above was repeated.

Protocol 3: Platelet counts were done on blood obtained directly from various organs of six hypothermic dogs (Numbers 11, 12, 13, 16, 17 and 18) by making small stab incisions. These counts were compared with counts performed on blood obtained simultaneously from the aorta by means of a polyethylene catheter threaded up the femoral artery.

Protocol 4: Biopsies were taken from skeletal muscles, skin, spleen, liver, kidney, lung, and heart of four animals (Numbers 11, 12, 13 and 17) at the height of hypothermia. These tissue sections were fixed in isotonic,

[1] Present address: Banco de Sangre del Distrito Federal, Caracas, Venezuela.

[2] Department of Experimental Surgery, Walter Reed Army Institute of Research.

TABLE I

Hypothermia in a dog (number 32) with radioactive platelets

	Platelets/ cu. mm.	Platelet radioactivity counts/platelet/ second
Before hypothermia	204,000	3.7×10^{-9}
Maximal hypothermia	58,000	
After hypothermia	225,000	3.0×10^{-9}

buffered formalin and stained with hematoxylin-eosin and Giemsa stains.

Protocol 5a: In three dogs (Numbers 11, 12 and 13) after anesthesia was induced but prior to cooling, two polyethylene catheters were set in place, one in the aorta by threading it up the femoral artery, and the other via the femoral vein in the inferior vena cava almost to the level of the right auricle. Samples of blood were taken from both catheters at intervals during the time of cooling and rewarming and counts were done. In a control experiment an animal was manipulated in the same manner except that hypothermia was not induced. The leukocyte and platelet counts did not significantly vary during the six hour period of manipulation and anesthesia (Table IV). This serves to demonstrate that the alterations of these counts which regularly occurred during hypothermia and rewarming were not due to such variables as time, anesthesia, trauma or the presence of catheters.

b. The procedure was that of protocol 5a except that the tip of the intravenous catheter was in the vena cava below the level of the renal vein in dogs Number 16 and 17.

c. As in protocol 5a but with three catheters; one in the aorta, one in low inferior vena cava, and one in the hepatic vein. The latter catheter was placed by direct observation during laparotomy. This was done in three dogs (Numbers 18, 19 and 20).

d. As in protocol 5a, but with catheters in the aorta, low inferior vena cava, hepatic vein, and in the portal vein just as it entered the liver. The latter two catheters were placed under direct observation during laparotomy. Because of the technical difficulties of this procedure only one animal was adequately studied, although the experiment was done five times (Dogs 21, 22, 23, 24 and 25).

Protocol 6a: Splenectomy was performed on two healthy dogs (Numbers 14 and 15) and they were allowed to recover from the operation. One week later protocol 5a was carried out.

b. Hepatectomy was performed on two healthy dogs (Numbers 26 and 27). The incision was closed and shortly thereafter they were cooled. In these experiments samples of blood were taken only from the aorta.

c. Combined splenectomy and hepatectomy were performed in four dogs (Numbers 28, 29, 30 and 31) and the animals were immediately cooled. Specimens were taken from catheters placed in the upper and the lower inferior vena cava, and from the aorta.

TABLE II

Platelet counts from incised wounds of various organs, done at maximum hypothermia (20° C.)

Dog no.	Aorta	Spleen	Liver	Lung
11	28,500	31,500	10,000	6,000
12	24,000	55,000	13,500	10,000
13	68,500	59,500	20,500	11,500
18	41,000	38,500	25,500	20,000

RESULTS

As previously reported, the platelet and leukocyte counts fell during the cooling and returned to the normal range when the animals were rewarmed. In general the changes in the leukocyte counts were not so great as the changes in platelet counts nor so consistent.

Protocol 1

Bone marrow aspirations and biopsies reveal no differences in the megakaryocytes seen before, during, or after the hypothermic state. Platelet formation and platelet morphology also appeared unchanged.

Protocol 2

Table I summarizes the results of the radioactive platelet transfusion. This table demonstrates that the platelets which appeared in the circulation following rewarming of the hypothermic dogs had 80 per cent of the radioactivity of the platelets present prior to hypothermia. In other words, 80 per cent of the radioactive platelets present prior to hypothermia reappeared on rewarming. An analysis of this matter has been published elsewhere (6).

TABLE III

Platelet counts in a dog (number 10) during deep hypothermia —a comparison of counts taken by catheter from the aorta and from incised wounds of the tongue

	Platelets/cu. mm.	
Temperature	Aorta	Capillaries (tongue)
(° C.)		
37	445,000	339,000
25	213,000	199,000
20	28,500	33,000
25	75,000	71,500
30	451,000	466,000

TABLE IV

Blood counts during deep hypothermia (dog number 13)—a comparison of counts from the aorta and high in the inferior vena cava

Temperature	Aorta			High vena cava		
	Platelets	Hemoglobin	Leukocytes	Platelets	Hemoglobin	Leukocytes
(° C.)	(per cu. mm.)	(Gm.%)	(per cu. mm.)	(per cu. mm.)	(Gm. %)	(per cu. mm.)
37	332,000	13.6	14,800	298,000	13.3	15,950
25	183,000	16.2	10,900	131,000	15.7	13,250
20	68,500	19.3	6,250	35,000	18.7	6,500
25	144,500	14.7	8,800	195,000	12.7	9,850
30	220,000	12.4	10,850	249,000	13.1	11,850
	Blood counts on control dog (no hypothermia)					
1 hour	387,000		36,600	393,000		36,000
2 hours	395,000		39,150	380,000		36,750
4 hours	357,000		37,150	420,000		33,800
5 hours	348,000		55,900	379,000		49,000
6 hours	376,000		50,000	385,000		47,100

Protocol 3

Table II demonstrates a similar decline in platelet counts obtained before and during deep hypothermia from aorta, spleen, liver and lung. The platelet counts of blood from the spleen and the lung tended to be lower than the others, but these experiments did not uncover any site of platelet storage. Blood from the cut tongue had changes in platelet counts that closely resembled those from the aorta (Table III).

Protocol 4

Biopsies taken from various organs at the height of hypothermia revealed no histological evidence of platelet storage.

Protocol 5a

Table IV demonstrates the results of platelet and leukocyte counts carried out simultaneously from catheters in the aorta and in the inferior vena cava proximal to the entrance of the hepatic vein. The drop in platelets high in the inferior vena cava preceded the drop in platelets in the aorta during the cooling process. During the warming phase, the rise in platelet counts from samples taken high in the inferior vena cava preceded the rise in the aortic samples. Platelets were apparently filtered out somewhere between the aorta and high in the inferior vena cava during cooling, and were being released from this site during rewarming. This would rule out the pulmonary circulation as the site of platelet sequestration.

b. Table V shows the results obtained when the tip of the catheter in the vena cava was lowered so that it no longer obtained blood from both the systemic and portal systems. During cooling the caval platelet and leukocyte counts did not fall more rapidly than the aortic counts.

c. In Table VI are the results from hypothermic dogs with catheters placed in the aorta, low inferior vena cava and hepatic vein. Platelet and leukocyte counts from the hepatic vein fell more rapidly than those from the other vessels. During rewarming the counts from the hepatic vein

TABLE V

Blood counts in a dog (number 17) during hypothermia—a comparison of counts from the aorta and low in the inferior vena cava

Temperature	Aorta		Low vena cava	
	Platelets	Leukocytes	Platelets	Leukocytes
(° C.)	(per cu. mm.)	(per cu. mm.)	(per cu. mm.)	(per cu. mm.)
37	299,500	15,000	342,500	13,000
25	123,000	8,600	117,500	9,900
20	48,500	8,000	47,500	8,800

TABLE VI

Blood counts during deep hypothermia (dog number 20)—a comparison of counts from the aorta, low in the inferior vena cava, and from the hepatic vein

Temperature	Platelets			Leukocytes		
	Aorta	Low vena cava	Hepatic vein	Aorta	Low vena cava	Hepatic vein
(° C.)		(per cu. mm.)			(per cu. mm.)	
37	374,000	336,000	363,000	7,000	5,900	6,000
25	91,000	108,000	74,500	2,600	3,000	2,000
20	48,000	42,500	30,000	1,400	1,800	1,200
25	177,000	199,500	201,000	2,300	3,700	3,900
30	163,500	226,500	189,000	3,800	5,000	5,500

increased more rapidly than those from other vessels.

d. In Table VII, the results of platelet and white cell counts from catheters in the aorta, low inferior vena cava, portal vein, and hepatic vein are compared. The changes in platelet count in the hepatic and portal veins preceded the changes in the aorta and low inferior vena cava when decreasing and also when increasing. Because the changes in the hepatic vein preceded those in the portal vein, it is evident that the liver plays a considerable role in platelet sequestration. However, because the changes in portal vein preceded those in the inferior vena cava and the aorta there may be other sites of sequestration in the portal circulation.

Protocol 6a

Table VIII demonstrates that splenectomy does not significantly alter the response of the platelets, leukocytes, or hemoglobin during hypothermia. Furthermore, a comparison of the aortic counts with those of the high inferior vena cava (Table IX) indicates that the portal circulation may still be the major site of sequestration, even after splenectomy, although only the difference at the 25° C. (on rewarming) level is within the 95 per cent confidence limit.

b. The effects of total hepatectomy on the platelet counts in hypothermia are shown in Table VIII. Because of the magnitude of the surgical procedures, these animals did not show the characteristic rise in hematocrit. These results indicate that while the liver plays some role in this phenomenon, it may not be the major site of sequestration.

c. Table VIII also shows the results of combined hepatectomy and splenectomy prior to hypothermia. Only one of the four dogs in this group showed the characteristic rise in hematocrit. Again this may be explained by the magnitude of the surgical procedures and the associated blood loss. In each of the four dogs, the platelet and white cell loss was less than that seen in animals which did not have hepatectomy-splenectomy. Table X shows that there was no longer any significant difference in counts obtained high in the inferior vena cava and those obtained lower in the vena cava.

TABLE VII

Blood counts in a dog (number 19) during hypothermia—a comparison of counts from the aorta, low in the inferior vena cava, portal vein, and hepatic vein

Temperature	Platelets				Leukocytes			
	Aorta	Low vena cava	Portal vein	Hepatic vein	Aorta	Low vena cava	Portal vein	Hepatic vein
(° C.)		(per cu. mm.)				(per cu. mm.)		
37	242,000	233,000	212,000	146,500	18,400	18,200	11,600	11,200
25	106,000	82,000	53,500	40,000	6,300	8,400	6,000	8,300
20	27,000	23,000	21,000	11,500	4,000		3,800	4,100
25	64,000	73,000	77,500	101,000	6,000	5,300	4,000	5,900
30	129,500	126,000	141,500	150,000	7,700	7,900	8,300	7,900

TABLE VIII

Blood counts in splenectomized and hepatectomized dogs during hypothermia *

Dog No. 15 Splenectomy

Temperature	Aorta			High vena cava		
	Platelets	Hct.	Leukocytes	Platelets	Hct.	Leukocytes
(° C.)	(per cu. mm.)		(per cu. mm.)	(per cu. mm.)		(per cu. mm.)
37	369,500	14.3	24,900	262,000	13.8	20,500
25	138,000	15.7	8,200	108,000	15.1	6,100
20	42,500	16.4	5,200	34,000	15.2	6,100
25	75,500	15.1	6,600	95,000	15.5	7,000
30	198,500	16.2	12,600	167,000	15.9	12,000

Dog No. 27 Total hepatectomy

Temperature	Platelets	Leukocytes	Hct.
(° C.)	(per cu. mm.)	(per cu. mm.)	
37	248,000	13,200	53.9
25	135,500	11,000	57
20	54,000	6,500	55.5
25	133,000	7,400	55.5
30	131,000	15,900	57.5

Dog No. 28 Splenectomy and total hepatectomy

Temperature	Platelets	Leukocytes	Hct.
(° C.)	(per cu. mm.)	(per cu. mm.)	
37	221,000	7,950	53
25	137,000	8,700	58
20	106,000	6,700	54

* All counts from Dogs 27 and 28 were done on blood from the aorta.

Figure 1 is a scattergram which summarizes the effects of organ excision on the platelet counts in hypothermia. Figure 2 is a scattergram of the white cell changes.

DISCUSSION

In dogs, most of the platelets and white cells disappear during hypothermia, and quickly reappear during rewarming. Are the platelets and white cells which reappear newly formed or are they simply the old platelets and white cells returning to the circulation after a period of sequestration caused by the hypothermia? The bone marrow studies support the sequestration theory. If the reappearance of platelets in the peripheral blood is due to new production, we would expect the megakaryocytes in the marrow to show evidence of increased activity. This was not found in the aspirates and sections of the marrow. The theory of platelet sequestration is further supported by the results of the experiment with platelets tagged with P^{32}. Since 80 per cent of the platelets tagged with P^{32} prior to hypothermia are still present following rewarming, the disappearance of platelets during hypothermia must be due to sequestration rather than to de-

TABLE IX

Blood counts in a splenectomized dog (number 14) during hypothermia—a comparison of counts from the aorta and high in the inferior vena cava

Temperature	Aorta			High vena cava		
	Platelets	Hemoglobin	Leukocytes	Platelets	Hemoglobin	Leukocytes
(° C.)	(per cu. mm.)	(Gm. %)	(per cu. mm.)	(per cu. mm.)	(Gm. %)	(per cu. mm.)
37	360,500	16.8	11,400	257,000	17.6	9,100
25	149,000	18.7	7,800	115,000	18.0	6,300
20	58,500	18.0	6,000	49,000	18.3	5,800
25	98,500	16.4	3,000	135,000	16.8	5,000
30	145,500	17.8	5,500	205,500	17.3	5,900

TABLE X

Blood counts in a dog during hypothermia following splenectomy and complete hepatectomy—a comparison of counts in the upper and lower vena cava (dog number 29)

Temperature	Upper vena cava			Lower vena cava		
	Platelets	Hct.	Leukocytes	Platelets	Hct.	Leukocytes
(° C.)	(*per cu. mm.*)		(*per cu. mm.*)	(*per cu. mm.*)		(*per cu. mm.*)
37	268,000	54	6,700	265,000	56	6,350
25	211,000	53	6,000	167,000	53	5,000
20	103,500	52	3,500	110,500	52	3,650
25	104,000	49	5,850	108,500	49	5,200
30	143,500	52	7,550	199,500	52	10,800

struction. As reported elsewhere (6), the P^{32} method of tagging platelets gives highly reproducible results. Counts per minute per platelet in successive aliquots of blood agree within 10 per cent. Very little, if any, of the radioactivity is contributed by contamination of the platelet samples by white blood cells or red blood cells, since these elements are nearly completely removed by differential centrifugation. Furthermore, when the donor blood is collected in glass vacuum bottles with ACD anticoagulant, the transfused platelets do not survive and no radioactivity can be detected in the platelets of the recipient three hours post-transfusion, which further demonstrates that red and white cell radioactivity play no role in the platelet radioactivity.

The site of sequestration was not revealed by biopsies of the various organs nor by samples of the capillary blood from these organs. The catheterization studies, however, did indicate where some of the sequestration occurs. During hypothermia, the drop in platelet counts high in the vena cava preceded the drop in the lower vena cava. During rewarming, the rise in platelets high in the inferior vena cava preceded the rise in the lower vena cava. Hepatic vein catheterizations indicated that these differences were due to dilution, with blood low in platelets from the portal circulation, of the samples obtained from the high vena cava. The contribution of the liver to the sequestration was demonstrated by placing catheters in the blood stream flowing into (portal vein) and out of (hepatic vein) the liver. However, since the platelet counts in the portal vein were less than those of the lower vena cava there must have been some sequestration of platelets elsewhere in the portal circulation.

The differences in platelet counts obtained by these catheterization studies in some cases are of small degree. However, the consistency of the direction of the changes suggests that they are significant. Brecher, Schneiderman, and Cronkite (7) have made a statistical evaluation of direct platelet counts by phase microsopy. When two separate pipettes and two separate chambers are used for each determination, the coefficient of variation is 8, and the 95 per cent confidence limits are $\pm$ 16 per cent. This figure includes both the pipette and the field error. Using this limit many of the platelet differences are significant. For example, in Table IV the differences between platelet count from the aorta and high inferior vena cava are significant at 25° C. (during cooling), 20° C., and 25° C. (during warming). In contrast, the differences between aorta and high inferior vena cava counts after splenectomy-hepatectomy (Table VIII, Dog 15) and in the control dog (Table IV) are not significant. The differences between high and low inferior vena cava platelet counts after splenectomy-hepatectomy are also not significant (Table X). The differences between aorta and hepatic vein counts (Table VII) are significant at the 25° C. (during cooling), 20° C., and 25° C. (during warming) levels. The differences between hepatic vein and portal vein are significant at the 20° C. and 25° C. (during warming) levels. However, the differences in platelet counts of Table V and VI, while consistent, are not within 95 per cent confidence limits except for the differences between aorta and hepatic vein counts at the 20° C. level, and between low inferior vena cava and hepatic vein at the 25° C. (during cooling) level.

The extirpation experiments indicated that the liver and spleen together were a major site of platelet sequestration. Removal of both organs

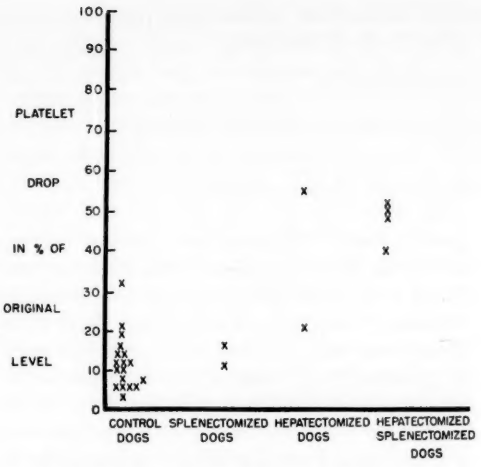

FIG. 1. RESPONSE OF PLATELETS TO HYPOTHERMIA

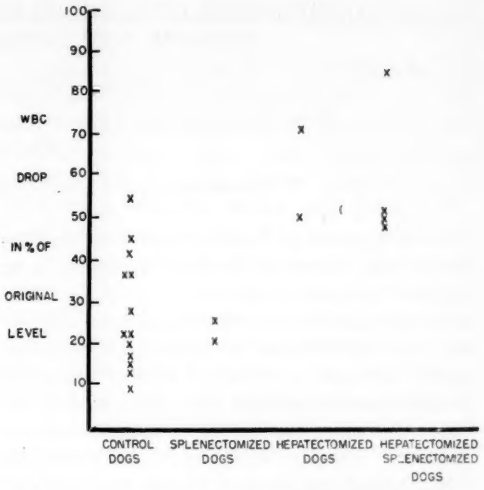

FIG. 2. RESPONSE OF LEUKOCYTES TO HYPOTHERMIA

had a greater effect than the sum of the effects obtained by removing each of them separately. Figure 1 summarizes the effects of organ removal on the thrombocytopenia of hypothermia. This diagram shows that hepatectomy and splenectomy decreased the thrombocytopenia of hypothermia but did not abolish it. There must be other sites of sequestration which we have not demonstrated.

The results of leukocyte studies, while they tended to parallel those of platelet studies, were much less consistent. Figure 2 summarizes the effects of organ removal on the disappearance of white cells.

SUMMARY

Investigations have been made into the mechanisms of the thrombocytopenia and leukopenia that occur in hypothermic dogs. By tagging platelets with P^{32}, the thrombocytopenia was shown to be caused by sequestration rather than destruction of platelets. This conclusion was supported by bone marrow studies which revealed no evidence of increased formation of platelets.

By sampling the blood in various vessels through polyethylene catheters it was demonstrated that the major site of platelet sequestration is in the portal circulation.

By extirpation it was shown that the liver and spleen play a major role in the sequestration. However, since hepatectomized-splenectomized dogs still develop some thrombocytopenia, other areas must also be sites of sequestration.

Changes in the leukocyte counts tended to parallel those of the platelets and were apparently due to similar mechanisms. However, they were not as consistent as those involving the platelets.

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COMPARISON OF VARIOUS PROCEDURES FOR DETERMINING SUCROSE AND INULIN SPACE IN THE DOG[1]

By H. L. WHITE AND DORIS ROLF

(From the Department of Physiology, Washington University School of Medicine, St. Louis, Mo.)

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Seven types of procedures have been used for determining volume of distribution (space) as an estimate of extracellular fluid (ECF). These are: (a) *infusion-recovery,* with infusion continuing until equilibration (as indicated by constant plasma level and by failure of further infusion to change space) is achieved (1); (b) *calibrated infusion* continuing until constant space is achieved (2); (c) *injection-extrapolation, i.e.,* extrapolation of falling plasma curve to zero time following a single injection in intact (3) or nephrectomized (4) subjects; (d) *simple dilution* spaces following a single injection as recovered minus excreted/plasma level (5), or as injected minus excreted/plasma level (6, 7); (e) *renal clearance/slope, i.e.,* following a single injection of substances which are completely recovered in the urine (renal clearance equals total clearance) and whose falling plasma curve is a straight semilog line, as renal clearance in a period/change in natural logarithm of plasma level in that period (8); (f) *total clearance/slope, i.e.,* following a calibrated infusion permitting calculation of total clearance, as total clearance/change in the natural logarithm of plasma level (9); (g) *differential clearance,* as amount excreted in a period/change in plasma level in that period, following a single injection (10).

With procedure (a), using a substance previously shown to be completely recovered, the space is calculated as amount recovered following termination of infusion/concentration in plasma water (ECF) shortly before termination. Equilibration is hastened by a priming injection. With procedure (b) a completely recoverable substance is infused without priming. Amount present in subject at time t is taken as amount infused minus amount excreted up to time t. This is divided by concentration in plasma water (ECF) to get space at time t. Equilibration of sucrose between plasma and ECF is practically achieved even while plasma level is still rising *slowly,* as is indicated by constancy of successive space values. Rate of infusion must be controlled with great accuracy. In procedure (a) infusion rate need not be accurately known, and a 5 per cent error in recovered amount introduces only a 5 per cent error in space, but a 5 per cent error in infusion rate in procedure (b) may cause an error of 8 per cent in space at 90 minutes, the error increasing with time and being greater the higher the subject's clearance. It is possible, but not easy, to keep the infusion rate error below 5 per cent.

Procedure (c) in intact subjects depends on the precarious assumption that after a single injection equilibration between plasma and some reproducible compartments of total ECF exists so long as the rapidly falling plasma curve follows a straight semilog line. The amount injected is divided by the zero time extrapolated plasma (ECF) level to get space. In practice, the falling plasma curve frequently fails to follow a straight semilog line. Procedure (c) in nephrectomized subjects is on a much sounder basis. Cell entry and metabolic destruction, which are known to occur with both inulin and sucrose (11, 12), are allowed for by the extrapolation procedure, and there is no significant error (inulin or sucrose) in taking single (simple dilution) values at one hour in rats and at one and a half to two hours in dogs. At this time equilibration between plasma and some reproducible compartments of ECF is practically achieved, as shown by the *very slowly* falling straight semilog line of plasma levels, while cell entry and destruction are still negligible.

Procedure (d) for simple dilution spaces as originally employed (6, 7) is subject to errors of two kinds. The first is represented by whatever error is contained in the assumption that equilibration between plasma and ECF can occur with

[1] Aided by grant No. A-92 C6, National Institutes of Health, United States Public Health Service.

a *rapidly* falling plasma level. The second is because with progressive destruction, even of only a few per cent an hour, cumulative and rapidly increasing percentage errors appear in the dividend presumed to represent the amount remaining in the subject, thus yielding erroneously high space values which increase with time. The attempt to circumvent this difficulty (5), by calculating space as total recovered minus excreted up to time t/plasma level at time t, depends on the inherently improbable assumption that destruction (or sequestration) is immediate. While this modification yields space values which do not show the rapid and progressive increases seen with the original procedure, the values are erratic, and our demonstration that spaces calculated with allowance for progressive destruction, as injected minus (excreted plus destroyed)/plasma level, are more nearly constant than those of Gilman, Philips, and Koelle (5) makes the assumption of immediate destruction unnecessary.

Poulos' expression (10) for procedure (g), space equals amount excreted in a period/change in plasma level in that period, is equivalent to the original expression (8), space equals renal clearance/slope of the line obtained by plotting natural logarithm of plasma level against time, here designated as procedure (e).[2] It was assumed by Newman, Bordley, and Winternitz (8) "that an equilibrium of concentration is established throughout the fluid." It is not apparent why their calculations are presumed to be valid by 25 minutes after injection, while in those of Poulos (10) a delay of 120 minutes is recommended, mannitol being used in both cases.

Procedure (f) was designed to permit use of substances whose clearance is partly extrarenal. Space equals total clearance in a period/change in natural logarithm of plasma concentration in that period (9). Obviously, only substances whose plasma concentration curves follow a straight semilog line after termination of infusion can be used; inulin is not suitable. Total clearance is obtained from the rate of *calibrated* infusion maintaining constant plasma level. Equilibration within the measured space is assumed to persist following cessation of the calibrated infusion.

Constancy of clearance is a prerequisite for injection-extrapolation in intact subjects (3), for renal or total clearance/slope (8, 9), and for differential clearance (10). Any simple dilution procedure (5, 6) must assume maintenance of equilibration between plasma and ECF. We therefore believe that the *infusion-recovery* and the *calibrated infusion* spaces should most nearly represent some reproducible compartments of total ECF in *intact* subjects, and that the *injection-extrapolation,* or the *simple dilution* value at one (rat) or one and a half to two (dog) hours after injection, should give equally valid values in *nephrectomized* subjects. In this paper we compare in a given dog the sucrose spaces believed to best represent the truly equilibrated space with those determined by other methods. Similar comparisons were made with inulin.

PROCEDURE AND METHODS

Comparisons were made on each of three female dogs. Five-hour recoveries following a single intravenous injection of saccharide were made on dogs without anesthesia or with Pentothal® Sodium; no effect of anesthesia on recovery was seen. Urine flows were elevated by water administration via stomach tube before beginning the experiment, with supplemental water doses in some experiments. Flows were 1.5 to 4 ml. per minute for the first two and a half hours or longer, since water diuresis is not affected by Pentothal® Sodium. An initial dose of about 10 ml. of 2.5 per cent Pentothal® Sodium intravenously was supplemented with doses of 1 to 4 ml. at about 30 minute intervals. Since urine flows were well maintained, no corrections for dead space have been made. Dogs were deprived of food but not water from the afternoon preceding the experimental day. Inuloid blank excretion in such fasted dogs is always close to 1.5 mg. per hour, and plasma inuloid levels are 11 to 15 mg. per liter, the sucrose equivalents being twice as high. Plasma inuloid blanks are 15 to 22 mg. per liter in control nephrectomized dogs at 24 hours, averaging 19 mg. Variations in postnephrectomy blank are a negligible percentage of the high 24 hour plasma levels produced in nephrectomized dogs (inulin about 1,000 mg. per liter, sucrose about 1,700).

Single injections of about 0.44 Gm. sucrose or 0.22 Gm. inulin per Kg. body weight were made from a syringe as 10 per cent sucrose in water or as 5 per cent inulin in saline, injection time being sometimes one minute and sometimes ten minutes. Residual saccharide in the beaker and syringe was determined and subtracted from the weighed amount to give the amount injected. For continuous or calibrated infusion, a weighed amount

[2] Newman (8): $Vc = \frac{C}{S} = \frac{UV/Pav}{(\ln Pa - \ln Pb)/(tb - ta)} = \frac{UV(tb - ta)}{Pa - Pb}$ [Poulos (10)].

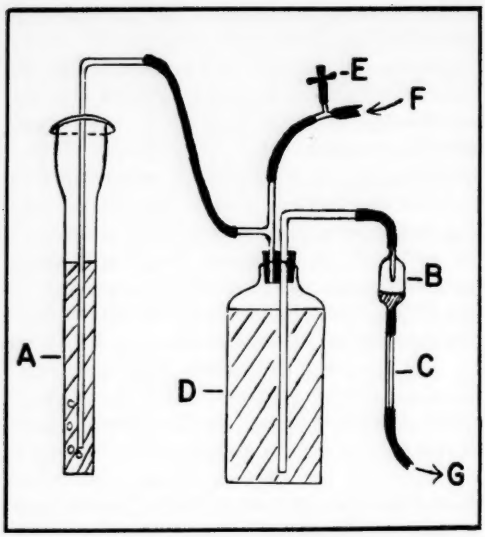

FIG. 1. INFUSION APPARATUS

A, mercury overflow valve; B, Murphy drip bulb; C, glass capillary; D, infusion bottle; E, clamp; F, from air line; G, to vein.

of saccharide, plus sodium chloride to make the solution isotonic, were dissolved in the injection bottle. The solution was infused, with the bottle pressure maintained at about 140 mm. Hg by a mercury overflow valve, at about 1.6 ml. per minute through a high resistance glass capillary inserted in the rubber tubing between the bottle and plastic tubing in the vein. A Murphy drip bulb in the injection line permitted a check on the constancy of infusion rate. The arrangement is illustrated in Figure 1. For the calibrated infusion determinations the drop rates in the bulb were calibrated against weighed amounts of solution delivered before and after the experiments. Drop size on a given day usually remained constant within plus or minus 1 per cent from the mean. Mean rate of saccharide infusion was determined as weighed amount of saccharide put into infusion flask minus residual amount determined by analysis, divided by the number of minutes. Constancy of infusion rate to plus or minus 2 or 3 per cent from the mean was controlled by drop counting. The rate of infusion per minute per Kg. body weight was about 4 mg. inulin and 8 mg. sucrose. A priming injection of about 0.22 Gm. inulin or 0.44 Gm. sucrose per Kg. body weight from a syringe preceded the continuous infusion in the infusion-recovery determinations.

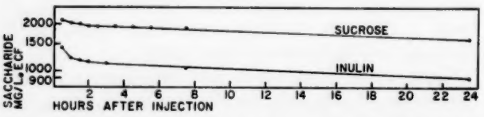

FIG. 2. CONCENTRATIONS OF SUCROSE AND INULIN IN PLASMA WATER FOLLOWING INTRAVENOUS INJECTION OF ABOUT 0.42 GM. SUCROSE OR 0.21 GM. INULIN PER KG. BODY WEIGHT INTO NEPHRECTOMIZED DOGS

Each curve shows average of three dogs.

For space determinations, as cited by other workers (3, 5, 6, 10), plasma levels were determined on samples drawn from a leg vein at various times after injection, with corresponding urine determinations. Venous plasma is presumed to be more nearly equilibrated with ECF than is arterial plasma. For determinations according to Gaudino, Schwartz, and Levitt (1), constancy of plasma level at about 1,000 mg. inulin and 2,000 mg. sucrose per liter was verified; this was attained at one and a half to two hours of infusion with sucrose and with inulin. For determinations according to Swan, Madisso, and Pitts (4), dogs were nephrectomized under barbital sodium and given about 0.22 Gm. inulin or 0.44 Gm. sucrose per Kg. body weight intravenously, blood samples being obtained at various times up to 24 hours. Resultant plasma levels are seen in Figure 2.

The bladder was washed twice with water at the end of each urine period. Sucrose and inulin were determined essentially according to Schreiner (13), using zinc-sodium hydroxide plasma filtrates. We have used sucrose obtained from a grocery store. Inulin was Pfanstiehl C. P. run through a Seitz filter, precipitated from 70 per cent alcohol and dried. It contained less than 1 per cent reducing substance. Standards were made from the material injected. Kidney invertase and inulinase activities were determined on kidney homogenates incubated at 38° C. for an hour; reducing substances after incubation were estimated on zinc-sodium hydroxide filtrates by comparison with known amounts of glucose or fructose similarly treated with qualitative Benedict's solution.

RESULTS

Recoveries of sucrose and inulin, and comparison of spaces determined according to previous workers (1, 3, 4, 6, 10) are shown in Table I. The simple dilution space (I–E)/P (injected minus excreted/plasma water level) at zero time (I/Po) is the injection-extrapolation space (3). Simple dilution spaces (I–E)/P determined according to Lavietes, Bourdillon, and Klinghoffer (6) and Elkinton (7) rise progressively in all experiments. Simple dilution spaces (R–E)/P (recovered minus excreted/plasma water level) determined according to Gilman, Philips, and Koelle (5) are not shown in the table. They were more nearly constant than was (I–E)/P, but were erratic, sometimes rising and sometimes falling.

TABLE I

*Five-hour recoveries of injected saccharides, and comparison of spaces with different procedures**

Date	Wt. Kg.	% Recovery in 5 hrs.	Inf.-Rec.	Simple dilution $\frac{I-E}{P}$ Minutes after end of injection or of infusion: 0	30	60	90	120	150	Simple dilution $\frac{I-(E+D)}{P}$ Minutes after end of injection or of infusion: 30	60	90	120	150	Differential clearance: 0–60	30–60	60–90	90–120	120–150	Overall
										Sucrose Dog 1										
10/10	10.4	94.1																		
10/24	10.5	92.5																		
11/23	12.1		2.76			3.29	3.40	4.69	5.84		2.94	3.06	3.01	2.67	2.36		2.40	2.77	3.11	2.64
12/13	11.8	86.0		2.71		3.36	3.88	4.84	6.30		2.93	2.88	2.93	2.92			2.48	2.34	2.26	2.39
1/3	12.1	90.1		2.74	2.74	2.96	3.38	4.14	5.57	2.65	2.62	2.58	2.60	2.63		2.39	2.37	2.27	2.25	2.35
	Average	90.7																		
1/17	12.3	Postnephrectomy space = 2.57 liters																		
										Sucrose Dog 6					0–30					
1/8	9.5	89.0		2.73	2.56	2.84	3.27	3.89	4.51	2.48	2.52	2.56	2.61	2.45		2.09	2.10	2.13	2.60	2.15
1/15	9.8			2.49	2.55	2.88	3.56	4.72	6.60	2.31	2.25	2.30	2.25	2.31		2.01	1.79	1.96	1.93	1.92
1/22	10.1	87.6		2.58	2.67	3.06	3.88	4.79	6.51	2.54	2.55	2.72	2.56	2.47		2.07	1.84	2.47	2.24	2.09
1/28	10.0	81.0																		
1/31	9.2		2.02																	
2/26	10.0	78.4		2.65	2.82	3.01	3.59	4.41		2.70	2.56	2.62	2.72				1.92	1.86		1.89
4/8	9.5		2.17		2.61	3.01	3.33			2.15	2.20	2.18			1.69	2.02	2.35			1.86
	Average	84.0																		
										Sucrose Dog 7										
2/28	12.9	91.3		3.04	3.24	3.36	3.65	4.08		3.16	3.09	3.12	3.16			2.62	2.77	2.74		2.69
3/6	12.6	87.8		3.38	3.42	3.50	3.82	4.36		3.10	3.01	3.04	3.10			2.38	2.79	2.61		2.54
3/14	12.2		2.60		2.84	3.23	3.46	3.53		2.60	2.60				2.09	2.24	2.65	3.25		2.33
	Average	89.6																		
										Inulin Dog 6										
3/5	9.6			2.90												2.04	2.09	2.12		2.07
3/12	9.7	93.0		3.11	2.59	2.84	3.43	4.26		2.42	2.36	2.38	2.38			1.83	2.00	2.07		1.92
3/18	9.5	93.8	1.96		3.42	4.32	5.36								1.38	2.15	2.44			1.66
4/1	9.3	91.0	1.86		3.02	2.93	3.34								1.24	3.18	2.34			1.63
	Average	92.9																		
4/19	9.4	Nephrectomy space = 1.73 liters																		
										Inulin Dog 7										
3/27	12.5	92.2		2.58	2.77	3.08	3.90	4.16		2.56	2.48	2.48				2.32	2.09	3.27		2.36
4/3	12.3															2.13	2.92	2.24		2.35
4/10	12.4	91.3	2.63		3.21	3.80	4.19								2.02	2.31	3.02			2.22
	Average	91.8																		
4/24	12.2	Nephrectomy space = 2.25 liters																		

*All spaces in liters. P = concentration in plasma water, mg./liter.
Inf.-Rec. = infusion-recovery space as (amount recovered at 5 hours/ave. % recovery at 5 hours)/P at equilibrium.
Simple dilution space as (I − E)/P at zero time = I/Po = injection-extrapolation space.
I − (E + D) = injected minus sum of excreted and destroyed.

TABLE II

Sucrose spaces on Dog 1 by infusion-recovery and calibrated infusion *

Date	Wt.	Calibrated infusion, minutes				Infusion-Recovery
		60	90	120	150	
	Kg.	*liters*				*liters*
12/20/56	12.3	2.59	2.68	2.69	2.66	2.76
1/10/57	12.2	2.36	2.53	2.63	2.69	2.58

* See references (1) and (2).

Neither sucrose nor inulin recovery was complete at five hours. For determinations according to Gaudino, Schwartz, and Levitt (1) we have therefore taken (amount recovered at five hours)/(average per cent recovery at five hours) as the amount of available material present in the dogs at cessation of infusion. In our experience recovery is never complete, with or without anesthesia; only 0.5 to 1.5 per cent additional inulin or sucrose is recovered in the two hours following the first five hours, and only a fraction of a per cent in the next 16 hours. This is consistent with our finding that plasma levels of inulin or sucrose, after blank correction, are only 6 to 12 mg. per liter at five hours. The unrecovered amount is either destroyed or remains in cells.

Table II shows calibrated infusion, and infusion-recovery sucrose spaces without priming, on Dog 1. Tables I and II show a surprising agreement in simple dilution values on Dog 1 (sucrose only) obtained as $I - (E + D)/P$ (see below), and by injection-extrapolation as $(I - E)/P$ at zero time, by differential clearance, by calibrated infusion, by infusion-recovery, and after nephrectomy. Even though the plasma curve was still rising at 150 minutes in the experiments of Table II (no priming), the infusion-recovery spaces agree well with that of 11/23, Table I, where constant plasma level was achieved (priming).

The differential clearance spaces of Dog 1 (Table I) on 11/23 were obtained on the falling plasma curve following cessation of infusion, *i.e.*, after equilibration. They rise progressively, as do both sucrose and inulin spaces so obtained on Dogs 6 and 7 on 4/8, 3/14, 3/18, 4/1 (except for the last period) and 4/10. If the differential clearance method is sound it should give results which are at least as consistent on a falling curve following equilibration as following a single injection. This procedure following a continuous infusion is essentially equivalent to the total clearance/slope procedure (9), since the amount destroyed in a period is with inulin a very small and with sucrose a small fraction of the amount excreted in that period, and the error is not cumulative. Thus, the amount leaving the subject in a period is represented closely by the amount excreted. Schwartz (9) pointed out that inulin was not suitable, since its falling plasma curve is not a simple exponential. Therefore, our results of 3/18, 4/1 and 4/10 with inulin are not surprising. We were disappointed, however, to find that the sucrose spaces of 11/23, 4/8 and 3/14 following equilibration by continuous infusion were actually less consistent than those following a single injection, even though observations in the latter case began as early as 30 minutes after injection. It appears to us that the frequent consistent results with the single injection differential clearance procedure (10) may be fortuitously due to the operation of opposing errors. Our failure to get the results with sucrose which would be expected from Schwartz' analysis (9) indicates failure to maintain equilibration between plasma and ECF following cessation of infusion.

In Dogs 6 and 7 the injection-extrapolation sucrose spaces fail to show the good agreement with infusion-recovery spaces observed in Dog 1. The injection-extrapolation inulin spaces on Dog 6 are much higher than the infusion-recovery or the postnephrectomy, although in Dog 7 the agreement is good. In Dogs 6 and 7 the differential clearance sucrose spaces following a single injection agree well with the infusion-recovery, and the differential clearance inulin single injection spaces agree fairly well with infusion-recovery and postnephrectomy.

The progressive rise in simple dilution space $(I - E)/P$ has been interpreted (5) as meaning that destruction is complete quite early after injection, and on this basis (recovered minus excreted)/plasma water level, $(R - E)/P$, was taken as a better expression. Since such immediate destruction seemed inherently improbable, we have made space calculations on the assumption that destruction is progressive. Destruction means disappearance from ECF other than

through excretion, whether due to actual destruction in ECF or cells, or to cell entry and trapping. The method of calculating rate of destruction was as follows:

Assume that the rates of excretion and of destruction are proportional to the concentration.[3] Let $k = (\ln C(o) - \ln C(t)/t$, where C is concentration of particles in the fluid compartment. $k = \epsilon + \delta$, where ϵ and δ are rate constants of excretion and destruction, respectively.

$$\epsilon = \frac{kN_\epsilon(t)}{N(o)} \cdot \frac{C(o)}{C(o) - C(t)},$$

where $N_\epsilon(t)$ is amount excreted in time t, N(o) is amount present in compartment at beginning of period; C(o) and C(t) are concentrations at beginning and end of period.

$$N\delta(t) = \frac{\delta}{k} \cdot N(o) \cdot \frac{C(o) - C(t)}{C(o)},$$

where $N_\delta(t)$ is the amount destroyed in time t.

The sum of cumulative excretion plus destruction up to the end of a given period is subtracted from the amount injected to give the amount of diffusible material remaining at the end of that period. This value is divided by the concentration in plasma water at that time to get the space. These spaces are shown in Table I under the heading $I - (E + D)/P$. This method can be employed only if fall in concentration follows a straight semilog line rather closely. In some cases this was true only for the early part of the experiment, in which case spaces could be calculated only for that part.

Plasma concentrations of early samples obtained at short intervals after the end of a single injection of 1 or 10 minutes' duration were always above the extrapolated values of the straight semilog line on which later samples fell. Values were on the line by 30 or 40 minutes, but attainment of a straight line cannot be considered as proof of equilibration, since 1.5 to 2 hours are required to reach a straight line after sucrose injection into a nephrectomized dog, and about 2 hours with inulin (Figure 2).

The rate of fall of plasma level following sucrose injection in a nephrectomized dog is considerably less than that expected from the rate of destruction evidenced by incompleteness of recovery from intact dogs. The recovery findings of Table I, together with the finding that not more than an additional 2 per cent is recovered after five hours, indicate that 10 to 15 per cent of the injected sucrose are destroyed at five hours. However, the plasma level five hours after injection in the nephrectomized dog is only 3.5 per cent lower than the zero time extrapolated equilibrium value (Figure 2), and this rate of fall persists for 24 hours. We have designated the rate of change per minute in the natural logarithm of plasma concentration as k; ϵ is that component of k due to excretion and δ that due to destruction. In nephrectomized dogs plasma sucrose level falls about 0.014 per cent per minute (Figure 2). In such dogs, $\delta = k = 0.00014$, *i.e.*, 0.014 per cent of the sucrose in a nephrectomized dog is destroyed per minute, and this value was quite constant in each of three dogs. Sucrose δ values in three intact dogs ($k - \epsilon$) range from 0.001 to 0.0022, with a mean of 0.00172, *i.e.*, 0.172 per cent of the sucrose in an intact dog is destroyed per minute, or about 12 times the rate in the nephrectomized dog. This discrepancy is far greater than can be accounted for by any errors of assumption, calculation or experiment. This indicates that a major part of the destruction depends on the presence of the kidneys.

To test this point, 2 per cent dog kidney homogenates in 10 per cent sucrose were incubated for one hour at 38° C., and zinc-sodium hydroxide filtrates tested for reducing substances. Blank homogenates showed essentially no reduction, while sucrose homogenates showed 5 per cent inversion of sucrose. Muscle homogenates showed no invertase activity. While quantitative comparison of the δ value (0.00086) obtained under these conditions with that calculated for intact dogs (0.00172) cannot be made, the observations show a high invertase activity of kidney tissue,

[3] This assumption is in error to the extent that equilibration between plasma and interstitial fluid fails to be complete. An error of 10 or 20 per cent here would introduce the same percentage error into amount calculated as destroyed. Since with sucrose our calculated amounts usually agreed within 10 per cent with observed amounts destroyed (see Appendix), this may be taken as indirect evidence that at about 30 minutes and thereafter, following a single injection into normal dogs, sucrose concentrations in plasma water and in pericellular water are usually not more than 10 per cent apart in cases where the falling plasma curve is following a straight semilog line.

and afford a plausible explanation for the much more rapid rate of sucrose destruction in the intact than in the nephrectomized dog. The glucose and fructose resulting from the inversion, which is assumed to take place in the tubular cells, presumably enter the blood stream, with subsequent tissue utilization of the glucose and of part of the fructose, and excretion of the unutilized fructose.

Under comparable conditions inulin was hydrolyzed by kidney homogenates to only about 15 per cent the extent observed with sucrose. Calculation of δ values for inulin has not been possible in most excretion experiments in intact dogs because of failure of the falling plasma curve to follow a straight semilog line. Where such calculations have been made they are in considerably greater error than with sucrose because the falling inulin curves always show some deviation from a straight semilog line and because rate of destruction is so low compared with rate of excretion. We can only say that δ values calculated for inulin are considerably lower than for sucrose, which is consistent with the finding that hydrolysis of inulin by kidney homogenates is considerably less than that of sucrose.

DISCUSSION

Our observed recoveries of sucrose are based on the resorcinol reaction after acid hydrolysis. Under the conditions employed (13), 99 per cent of the color of hydrolyzed sucrose is due to fructose. Since part of the resorcinol-reactive material in our urines is fructose, the actual sucrose recoveries are lower than given in Table I. Thus, Dog 6 on 1/28, after injection of 4.4 Gm. of sucrose, excreted 2,450 mg. of "sucrose" (resorcinol reaction) in the first hour and 826 mg. in the second hour. The urines were free from glucose by test with Clinistix Reagent strips (Ames Co., Inc., Elkhart, Indiana). The first hour's urine contained 100 mg. of the reducing substance, fructose. The second hour's urine contained 25 mg. of fructose. While 3,276 mg. of the injected sucrose appeared as resorcinol-reactive material in the first two hours, this consisted of 125 mg. fructose with the chromogenic activity of 250 mg. sucrose, and 3,026 mg. sucrose. Thus, only 92 per cent of the resorcinol-reactive material was sucrose, and if the experiment shows 90 per cent recovery of resorcinol-reaction material in five hours, only 83 per cent is unhydrolyzed sucrose. This example is a typical result. Our findings agree with those of Power and Keith (14). Incompleteness of sucrose recovery need not invalidate its use in space measurements, since its rate of destruction can be calculated and appropriate allowance made. Nor does the finding that a few per cent of the material determined as sucrose in the urine is fructose invalidate the space findings. No error is introduced into the excretion findings, and whatever small amounts of free fructose may exist in ECF must have about the same volume of distribution as sucrose. It is true that these changes make any simple method of calculating sucrose space in the dog invalid, but if the reports of others that sucrose is completely recovered from man are correct, these complications of calculations would not occur in experiments on man.

We were encouraged by the findings on Dog 1 to believe that any of the methods, except $(I - E)/P$, would give valid sucrose spaces. The findings on Dogs 6 and 7 do not support this optimism. Each procedure is discussed below.

Simple dilution space, $(I - E)/P$

A progressive rise is seen in all experiments with both sucrose and inulin. This is largely because the dividend is erroneously high by a progressively increasing percentage when destruction is not allowed for. For reasons stated above, we do not consider $(R - E)/P$ an adequate formula.

Simple dilution space, corrected for destruction, $I - (E + D)/P$

When allowance is made for material destroyed (or trapped), as well as for that excreted, dilution spaces remain reasonably constant on a given day, but in Dogs 6 and 7 are usually considerably higher than the infusion-recovery or postnephrectomy space. Since the assumption of progressive destruction is more probable than that of immediate destruction, and since spaces calculated on the former assumption are more nearly constant than those calculated on the latter, there seems to be no reason to retain the concept of immediate destruction. Nevertheless, we cannot

accept the $I - (E + D)/P$ spaces without reservation, since we doubt that equilibration is ever attained following a single injection. It may seem illogical to lack confidence in results obtained by a single-injection procedure even though they may agree with those obtained by the infusion-recovery, calibrated infusion, and postnephrectomy procedures. Our reasons are two. First, in our hands such agreement is not obtained *consistently*. Second, we believe the latter three methods are on a sounder, although admittedly not unassailable, theoretical basis.

Walser, Seldin, and Grollman (15), using radioactive inorganic sulfate, modified the injection-extrapolation procedure by dividing $I - E$ at 18 minutes in the dog, or 25 to 30 minutes in man, by concentration in interstitial water to get simple dilution spaces at those times. Demonstration that falling plasma curves become simple exponentials at such times is taken as evidence of equilibration between plasma and "functional extracellular fluid." [4]

The findings on the nephrectomized dog afford the strongest argument against such early equilibration. If such equilibration were in fact attained at about 18 minutes following a single rapid injection of radiosulfate in the intact dog, the initially rapidly falling curve following a single injection in the nephrectomized dog should "break" to the more slowly falling straight semilog line at about that time, whereas the change does not occur until one and a half to two hours, about the same time as for mannitol, inulin, sucrose and thiosulfate (4). It might be argued that the more rapidly diffusing substances equilibrate rapidly with the "functional" extracellular fluid, and that the last one to one and a half hours required to reach the straight semilog line is the time during which the less easily accessible connective tissue and "transcellular" compartments are being penetrated. If this were true, the radiosulfate and thiosulfate curves should have more nearly approached the extrapolated straight line at 30 minutes than should inulin, but there is no evidence in the findings of Swan, Madisso, and Pitts (4) that this is happening, although it is true that in our Figure 2 the sucrose curve is closer to the line at 30 minutes than is inulin. We can only say that while radiosulfate as employed by Walser, Seldin, and Grollman (15) may measure pericellular or "functional" ECF, the infusion-recovery, calibrated infusion and postnephrectomy procedures seem to us at present to be on a sounder theoretical basis.

Amount injected/plasma level extrapolated to zero time, I/Po, injection-extrapolation

With Dogs 6 and 7 this space, both sucrose and inulin are considerably higher than the infusion-recovery or the postnephrectomy. In many experiments no value can be obtained with this method because of uncertainty of slope even after the points are falling fairly closely on a straight semilog line. The finding that even with the best straight lines these values are higher than infusion-recovery or postnephrectomy spaces is further evidence that equilibration does not occur. Further difficulties in this method are discussed by Chesley and Lenobel (16).

Evidence that equilibration is not attained following a single injection may be summarized here. First, with both sucrose and inulin the injection-extrapolation space, when obtainable, is higher than the infusion-recovery or the postnephrectomy space. Second, simple dilution sucrose spaces after allowing for destruction, even though constant, are usually higher than infusion-recovery or postnephrectomy. Third, a straight semilog line of falling plasma sucrose level is inadequate evidence of equilibration, because the straight line, when seen, is achieved in 30 or 40 minutes following a single injection in the intact dog, while 1.5 to 2 hours are required in the nephrectomized dog. Fourth, Schachter, Freinkel, and Schwartz (17) have shown that equilibration of inulin is not achieved following a single injection in dog or man.

Failure of ourselves and others to get *consistent* agreement of space determinations by injection-extrapolation or simple dilution with values obtained by the infusion-recovery or postnephrectomy procedures, even when in the simple dilution procedure correction for destruction is made and constant successive values may be ob-

[4] Since rate of disappearance from plasma through cell entry and/or metabolism is about as great as that through excretion, simple dilution spaces as $(I - E)/P$ show the expected rapid and progressive increase with time.

tained, is interpreted as indicating failure of equilibration with the former procedures even when a more rapidly diffusing substance than inulin, *i.e.*, sucrose, is used. None of the considerations of this or the preceding paragraph constitutes proof that a satisfactorily close approach to equilibration cannot occur following a single injection of a still more rapidly diffusing substance, such as radiosulfate. We cannot dismiss the possibility that such a procedure (15) may measure pericellular plus some fraction of connective tissue fluid, at least in normal subjects. More repeated determinations on the same subjects are needed to establish degree of consistency.

Amount excreted in a period/change in plasma level in that period—Differential clearance

Poulos (10) has proposed an amount excreted in a period/change in plasma level in that period as a measure of volume distribution. This procedure does not demand that equilibration between plasma and interstitial fluid be achieved following a single injection, but does demand a constant difference between plasma and interstitial fluid levels. He postulated that the plasma and interstitial fluid curves do in fact become parallel at one and a half to two hours after a single injection. Even though the absolute values of differences between the curves become small as the two levels themselves become small, there is no theoretical reason to believe that the difference becomes a smaller percentage of the absolute values. Direct evidence, similar to that of Schachter, Freinkel, and Schwartz (17), is difficult or impossible to obtain on working with the low plasma levels existing late after a single injection.

Infusion-recovery procedure

We have not obtained complete recoveries following a single injection of either sucrose or inulin from the dog in 24 hours. This is true without anesthesia, or with the dog under Pentothal® Sodium for the first 5 or 6 hours. Recoveries of sucrose (resorcinol-reactive material in urine) at 5 hours averaged 90.7, 84.0 and 89.6 per cent in these three dogs; 98 to 99 per cent of that excreted in 24 hours have been excreted at 5 hours, 1 to 1.5 per cent more in the period from 5 to 7 hours, and none or a few tenths of a per cent between 7 and 24 hours. Recoveries of inulin at 5 hours averaged 92.9 and 91.8 per cent in Dogs 6 and 7; 99.5 per cent of that excreted in 24 hours has been excreted at 5 hours, and the remainder in the period from 5 to 7 hours. We have therefore taken amount excreted in five hours following cessation of infusion/average percentage recovery of a single injection in five hours as the diffusible or available amount remaining in the dog at end of infusion. This amount divided by the equilibrium concentration in plasma water gives the space. We consider this, together with the calibrated infusion (2) and the postnephrectomy values, as the best measures now available. This view is not negated by our finding on nephrectomized rats (12) that whole body inulin and sucrose spaces rise with time, following a straight semilog line rather closely for many hours; determinations were not made at short enough early intervals to establish the "break" in falling plasma levels. We are now convinced, on the basis of our and others' findings with the calibrated infusion and postnephrectomy procedures, that in the dog equilibration between plasma and interstitial (plus some fraction of connective tissue) fluid is achieved with both sucrose and inulin at two hours of a constant infusion preceded by a priming injection. Whatever cell entry, with destruction or trapping, has taken place within this time does not affect the result, since it is not seen in the recovered material.

Calibrated infusion procedure (2)

We have used this procedure, with sucrose only, in one dog (Table II). It agreed well with the infusion-recovery and postnephrectomy values. The practical handicap to its use is the necessarily high degree of precision in infusion rate. If destruction or trapping of the injected material is occurring, allowance must be made for this to get the best results, but we do not see how to do this with a plasma curve of the type seen in these experiments.

Although plasma levels are still rising slowly at two and a half hours of calibrated infusion (no priming), infusion-recovery values obtained after cessation of such infusion agree with those obtained following establishment of constant plasma

levels (priming). This finding may be added to the constancy of successive calibrated infusion spaces as further evidence that almost complete equilibration occurs at or before two hours with either method. Practical achievement of equilibration can occur because the percentage rate of rise of plasma after one and a half hours is low, a quite different situation from the high percentage rate of fall after a single injection or after termination of an infusion.

Postnephrectomy space

Our rate of fall of plasma levels following single injection into the nephrectomized dog is about 0.014 per cent per minute for sucrose and 0.016 per cent per minute for inulin for the period between 3 and 24 hours ($k = \delta = 0.00014$ and 0.00016). These values are lower than those of Swan, Madisso, and Pitts (4), who found k from 0.00025 to 0.0004 for sucrose, and 0.00036 to 0.00056 for inulin. Spaces are calculated as amount injected/concentration in plasma water extrapolated to zero time.

Our inulin k value agrees well with the 0.00012 of Finkenstaedt, O'Meara, and Merrill (18) and the 0.00014 of Bálint and Forgács (19), calculated from their data on nephrectomized dogs. Mulrow, Oestreich, and Swan's Figure 1 gives a k value of 0.00021 for sucrose (20). We have reported data (11, 12) yielding k values of about 0.0006 for inulin and 0.0005 for sucrose in nephrectomized rats, *i.e.*, both inulin and sucrose enter cells much more rapidly in nephrectomized rats than in nephrectomized dogs. This may mean greater activity of macrophages toward inulin in rats than in dogs. These k values are for whole rats; there is great variability among different tissues (11, 12).

A few words must be said against the possibility that these are abnormal results, *i.e.*, that cellular permeability to these saccharides is increased after nephrectomy. In the rat we have shown (12) that the rate of fall of plasma inulin level and of rise in calculated space (k value) is not different in rats nephrectomized 24 hours before injection from that in rats nephrectomized at time of injection. If cellular permeability to inulin is increased after nephrectomy, the increase should be greater the longer the animal has been nephrectomized. We think it probable that cell entry of inulin is largely through ingestion by macrophages, since k values are highest in skin, liver and spleen, tissues which are rich in macrophages; this view is supported by Jancsó's histochemical demonstration of inulin in such cells in connective tissue and liver of mice (as cited in Reference 19). The maintenance of a straight semilog line of falling plasma level in nephrectomized subjects in itself speaks against increasing cell permeability, as well as a against (a) appreciable continued diffusion (after the first hour in the rat and second hour in the dog) into less easily accessible extracellular compartments, and (b) actual increase in volume of ECF. Corresponding and additional evidence is presented that in the dog cellular permeability to inulin is not increased at several days after nephrectomy (19), or to sucrose at 30 hours (20).

Infusion-recovery spaces are somewhat higher than corresponding postnephrectomy spaces. This is largely accounted for by the positive water load existing at cessation of infusion. Intake up to cessation of infusion exceeded urine output by 0.09 to 0.25 liters. When correction is made for this, the infusion-recovery values are: 2.67 liters for Dog 1 sucrose on 11/23; 1.78 and 1.62 liters for Dog 6 inulin on 3/18 and 4/1; and 2.38 liters for Dog 7 inulin on 4/10. These agree well with the corresponding postnephrectomy spaces (Table I).

SUMMARY

Comparisons of various procedures for determining sucrose and inulin spaces in dogs have been made. The calibrated infusion, constant infusion to equilibration, and postnephrectomy values agree and are considered to be the most reliable. No single injection method in intact subjects is considered to be acceptable without reservation, even though values agreeing with the above methods are often obtained. The progressive rise in simple dilution space following a single injection, or cessation of constant infusion, of a substance not completely recoverable is ascribed to progressive rather than to immediate destruction or trapping. Expressions are given for calculating such destruction. Added evidence that diffusion equilibrium is achieved within two hours of constant infusion preceded by a priming injection

is afforded by the finding that infusion-recovery spaces so obtained agree with those obtained following cessation of a two hour infusion not preceded by priming, even though in the latter situation the plasma level is still rising slowly at two hours.

Since recoveries following injection of known amounts were never complete with either sucrose or inulin, we have taken the amount recovered in five hours/average per cent recovery in five hours as the value to be divided by the plasma water level at equilibrium to give the infusion-recovery space. This is believed to be the most practical valid method now available for use in intact subjects.

The major part of the destruction of sucrose following intravenous injection in the dog is made possible through inversion in the kidneys.

APPENDIX

Assume that the concentration, C(t), of sucrose in the fluid compartment is falling due to two processes only (excretion and destruction) and that the rate of each is proportional to C(t). These assumptions lead to the equations:

$$(1) \qquad \frac{dN_\epsilon(t)}{dt} = EC(t) = \frac{E}{V}N(t) = \epsilon N(t),$$

$$(2) \qquad \frac{dN_\delta(t)}{dt} = DC(t) = \frac{D}{V}N(t) = \delta N(t),$$

where V is the (constant) volume of the fluid compartment, and N(t) is amount of sucrose it contains at time t. Since only these processes are occurring, the rate of decrease of sucrose in the fluid compartment is the sum of these rates and we have

$$(3) \qquad -\frac{dN(t)}{dt} = kN(t) = \frac{dN_\epsilon(t)}{dt} + \frac{dN_\delta(t)}{dt} = (\epsilon + \delta)N(t),$$

and $\epsilon + \delta = k$ as defined in text.

To determine ϵ, we multiply Equation (1) by dt and integrate over time from 0 to t.

$$(4) \qquad \int_0^t dN_\epsilon(t) = \int_0^t \epsilon N(t)\,dt.$$

The left side becomes $N_\epsilon(t)$, which is the amount of sucrose excreted up to time t, since $N_\epsilon(o) = 0$. Also $N(t) = N(o)e^{-kt}$ since N(t) decreases exponentially with rate constant k.

$$(5) \qquad N_\epsilon(t) = \int_0^t \epsilon N(o)e^{-kt}\,dt.$$

$$(6) \qquad N_\epsilon(t) = -N(o)\frac{\epsilon}{k}e^{-kt}\Big]_0^t.$$

$$(7) \qquad N_\epsilon(t) = N(o)\frac{\epsilon}{k}(1 - e^{-kt}).$$

Using the fact that $\frac{C(t)}{C(o)} = e^{-kt}$ and rearranging, we have

$$(8) \qquad \epsilon = k\frac{N_\epsilon(t)}{N(o)}\cdot\frac{C(o)}{C(o) - C(t)}.$$

ϵ can be determined from this equation if the amount and concentration of sucrose in the fluid compartment (giving N(o), C(o), C(t) and k) and the amount of sucrose excreted $N_\epsilon(t)$ are determined at two or more different times. When k and ϵ are known, δ can be determined from $\delta = k - \epsilon$. When δ is known, the number of particles destroyed at any time t can be obtained from

$$(9) \qquad N_\delta(t) = \frac{\delta}{k}N(o)\frac{C(o) - C(t)}{C(o)},$$

which is derived as was Equation (7).

These expressions can be employed only during such time as the falling plasma level follows a straight semilog line, which is not attained until the end of the first urine period of 30 or 40 minutes' duration. We have, therefore, calculated ϵ from the beginning of the second urine period through such time as the line remains straight. For this calculation take C(o) as concentration at beginning of second period and N(o) as amount injected minus amount excreted in first period, *i.e.*, for this first calculation of ϵ, destruction in first period is neglected. This first approximation of ϵ is subtracted from k to get δ, and, using this value of δ, the amount destroyed in the first period is calculated as follows:

Mean interstitial fluid level during first period ($\underline{C}$) is assumed to be one-half of plasma level extrapolated to zero time. For calculating amount destroyed in first period, $N_\delta(t) = \frac{\delta}{k}\cdot N(o)\cdot\frac{C(o) - C(t)}{C(o)}$ cannot be used, since C(o) (here taken as concentration in interstitial fluid at beginning of first period) is not known, and since change in concentration does not follow a straight semilog line during first period. Instead, take $N_\delta(t) = \delta\underline{C}Vt$, where V is volume of interstitial fluid (taken as infusion-recovery space minus assumed plasma volume), and t is duration of first period in minutes.

This first approximation of $N_\delta(t)$ is subtracted from original N(o), and a second value for ϵ is calculated, using the new N(o). Subtracting the second ϵ from k gives the second approximation of δ. Using this δ, a second approximation of amount destroyed in first period is obtained. This process is continued until values of ϵ obtained in consecutive calculations agree closely. These values oscillate decrementally about a limit which is closely approached after four to eight steps; the mean of the last two values is used.

For calculation of amount destroyed in periods after the first, the expression $\frac{\delta}{k}\cdot N(o)\cdot\frac{C(o) - C(t)}{C(o)}$ is used, where N(o) is amount remaining, *i.e.*, injected minus (excreted plus destroyed), at beginning of period, and C(o) and C(t) are concentrations in plasma water at beginning and end of period. We are indebted to Dr. William Sleator, Jr. of this department for the above treatment.

The amounts of sucrose so calculated as being destroyed in the first five hours usually agreed within 10 per cent with the amounts which failed to be recovered in five hours; the maximum discrepancy was 20 per cent. With inulin the percentage discrepancies were greater, ranging from 10 to 33 per cent of the 6 to 8 per cent which failed to be recovered.

Our sucrose values of δ (change in the natural logarithm of C per minute due to destruction) for intact dogs average 0.00172 and for nephrectomized dogs 0.00014, giving extrarenal clearances of about 21 ml. per Kg. per hour for intact and 1.8 for nephrectomized dogs.

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THE ABSORPTION OF GLYCINE AND ITS CONVERSION TO SERINE IN PATIENTS WITH SPRUE

By C. E. BUTTERWORTH, Jr.,[1] RAFAEL SANTINI, Jr.,[1] and ENRIQUE PEREZ-SANTIAGO[2]

(From the United States Army Tropical Research Medical Laboratory, San Juan, Puerto Rico)

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Glycine has been used for many years as an orally administered test substance in the study of intestinal absorption. Most authors have utilized a technique measuring the total amino acid nitrogen of the blood at intervals after an oral dose of glycine, with the assumption that increases reflect the amount of glycine absorbed. Althausen, Doig, Uyeyama, and Weiden (1) have demonstrated the validity of this technique, but nevertheless concluded that absorptive tests for proteins and fats generally indicate greater impairment of absorption than do nutritional balance studies (2).

There have been conflicting reports concerning the absorption of glycine in patients with sprue, although recent work suggests impaired absorption of virtually every normally absorbable substance, including sodium chloride (3). In 1935, Heath and Fullerton (4) noted similar curves of blood amino acid nitrogen after either oral or intravenous administration of glycine to patients with impaired absorption. They noted that about 75 per cent of an injected dose of glycine disappeared from the blood within four or five minutes. They concluded that glycine is without value in studying the absorptive capacity of the intestine. However, Erf and Rhoads (5) in 1940 presented evidence suggesting that glycine is absorbed from the gastrointestinal tract more slowly in patients with sprue or pernicious anemia than in normal subjects. Evidence of this abnormality was not demonstrable in the same patients after remission was induced with liver extract. Drew, Dixon, and Samuel (6) found satisfactory absorption of glycine in five patients with sprue in a convalescent state.

In the investigations which form the basis of this report chemical determinations of glycine and serine were carried out, in addition to the usual total amino acid nitrogen determination, in plasma from normal subjects and patients with sprue. This procedure was undertaken because of the role of folic acid in the conversion of glycine to serine and because of presumptive evidence that tropical sprue patients are deficient in this vitamin. The belief that such patients may be deficient in folic acid stems from observations that: 1) they show prompt clinical improvement with the sole addition of folic acid to a protein-poor, vitamin-deficient diet (7), and 2) there is a greatly diminished urinary excretion of folic acid by sprue patients (8). Das Gupta, Chatterjea, and Basu (9) believe that nutritional macrocytic anemia, sometimes considered as synonymous with sprue, results primarily from a deficiency of folic acid.

It was postulated at the beginning of the study that impaired conversion of glycine to serine might lead to an accumulation of glycine in plasma, thus masking reduced absorption. In addition, the technique described might reveal other changes in the glycine and serine components not recognizable when only the total amino acid nitrogen is measured.

MATERIAL AND METHODS

The diagnosis of "tropical" sprue in relapse was based on the clinical presence of chronic diarrhea, anorexia, weight loss, glossitis, and megaloblastic anemia along with demonstration in the laboratory of impaired absorption of two or more test substances usually D-xylose, vitamin A, and dietary fat (10). Patients referred to as treated were those who had previously been studied during relapse, or who gave definite historical evidence of such a relapse with response to specific therapy. Treatment with folic acid or vitamin B_{12} was effective in correcting the anemia and bringing about great clinical improvement in all these subjects. However, eight of the nine continued to have episodes of diarrhea and displayed malabsorption for one or more test materials.

[1] Present address: Walter Reed Army Institute of Research, Washington, D. C.

[2] Present address: Bayamon District Hospital, Bayamon, Puerto Rico.

The patient who did not complain of chronic digestive symptoms showed persistent malabsorption of vitamin A.

Fasting subjects were given orally 25 Gm. doses of glycine freshly dissolved in 250 ml. tap water. This was followed by another 250 ml. tap water added to the same glass to ensure that the entire dose was received. Heparinized blood samples were collected immediately before administration of the dose, and one, one and one-half, two, three, and four hours after the dose. Plasma was separated within 20 minutes and proteins were precipitated by the addition of sodium tungstate and sulfuric acid. Glycine was determined by the method of Alexander, Landwehr, and Seligman (11); serine was determined by the method of Neidig and Hess (12) and total amino acid nitrogen was measured by a modification of the Folin and Wu method (13).

It was found that sugars normally present in blood interfere with the Neidig and Hess procedure, which involves the oxidation of serine to formaldehyde using periodic acid. In order to adapt the procedure to the measurement of serine in human plasma tungstic acid filtrates were passed through a column of cation exchange resin which retained serine but not the sugars. The following technique proved satisfactory: 5 ml. of blood plasma was precipitated with 5 ml. of 10 per cent sodium tungstate and 40 ml. of 0.083 N sulfuric acid. Twenty ml. of the filtrate was passed through a column containing 3 Gm. of Amberlite IR-120 in the hydrogen form. The column was washed with 30 ml. of distilled water. Elution of serine was accomplished by passing 25 ml. of 2 N sodium hydroxide through the column. The concentration of serine in the eluate was then determined by the Neidig and Hess procedure using all glass distilling equipment. The concentration of serine in the fasting plasma of 20 normal subjects (10 male, 10 female) was found to be 11.1 ± 2.2 micrograms per ml. This value is in close agreement with the findings of Stein and Moore (14) who reported a value of 11.2 micrograms per ml. An average of 97.8 per cent of added serine was recovered in a series of recovery tests.

In every case chemical determinations were completed the same day the dose was given, usually within five hours after collection of the sample. It has been found that erratic results will be obtained, presumably due to proteolysis, unless the plasma is precipitated promptly. Aliquots of pooled plasma samples stored at $-20°$ C. and tested at intervals during the course of a year showed wide variation in results. Protein free filtrates, however, under similar conditions showed satisfactory reproducibility on repeated testing.

For intravenous administration, glycine was added to pyrogen-free normal saline solution to make a 5 per cent glycine concentration and was then autoclaved. Doses were calculated on the basis of hematocrit and estimated blood volume so that comparable doses were given to all subjects. This dose (1 to 2.0 Gm.) would yield a concentration of 500 micrograms per ml. if immediately injected and yet would leave measurable quantities at the end of the 10 minute infusion period allowing for the rapid rate of plasma clearance. Samples were collected immediately after the injection, and 5, 15, 30, and 60 minutes after the end of the injection.

RESULTS

The plasma concentrations of glycine, serine and total amino acid nitrogen following oral administration of glycine are shown in Tables I, II, and III. In view of the fact that the 25 Gm. oral dose was greater in proportion to body weight for the subjects with sprue, it was decided to apply a correction factor. The average body weights were as follows: normal, 161 lbs. (range, 117 to 190); treated sprue, 112 lbs. (range, 101 to 125); untreated sprue, 99 lbs. (range, 75 to 112). A compensatory factor was present in the untreated subjects who were all anemic and therefore did not have much net reduction in plasma volume. Accordingly, values were all corrected to a normal weight and hematocrit by the following formula:

$$\text{Corrected value} = \left[(\text{uncorrected value} - \text{fasting value}) \times \frac{\text{weight}}{150} \times \frac{\text{100-hematocrit}}{55}\right] + \text{fasting value.}$$

This offers a somewhat more valid comparison without altering the pattern of results. The corrected value was used in the preparation of graphs.

TABLE I

Plasma glycine after oral administration of 25 Gm. glycine (mg. glycine per 100 ml. plasma)

	Nine normal subjects			Nine treated sprue subjects			Eight untreated sprue subjects		
	Mean	S.D.	Corrected	Mean	S.D.	Corrected	Mean	S.D.	Corrected
Fasting	1.3	0.2	1.3	2.0	0.4	2.0	1.7	0.4	1.7
1 hr.	22.4	5.4	23.9	28.0	10.2	21.2	11.2	4.5	10.5
1.5 hrs.	22.3	3.3	23.9	30.7	9.8	23.8	14.8	4.5	13.9
2 hrs.	17.3	2.5	18.4	29.9	13.1	22.9	18.3	5.2	17.1
3 hrs.	10.0	3.9	10.7	24.1	9.4	18.6	20.0	4.6	18.7
4 hrs.	6.1	1.3	6.5	17.0	7.5	14.8	18.7	6.0	17.6

TABLE II

Plasma serine after oral administration of 25 Gm. glycine (mg. serine per 100 ml. plasma)

	Nine normal subjects			Nine treated sprue subjects			Seven untreated sprue subjects		
	Mean	S.D.	Corrected	Mean	S.D.	Corrected	Mean	S.D.	Corrected
Fasting	1.1	0.2	1.1	1.1	0.5	1.1	1.9	0.6	1.9
1 hr.	2.8	0.5	2.9	2.4	0.6	2.0	2.7	0.8	2.5
1.5 hrs.	3.8	0.8	4.0	3.3	0.8	2.8	3.9	0.9	3.5
2 hrs.	3.8	1.0	4.0	4.0	1.1	3.3	3.9	1.1	3.5
3 hrs.	3.0	0.7	3.1	4.5	1.3	3.7	5.4	1.5	4.7
4 hrs.	2.3	0.4	2.4	3.8	1.5	3.1	5.5	1.8	4.8

The mean plasma values of glycine after oral administration of glycine are shown graphically in Figure 1. Plasma serine values determined at the same time are shown graphically in Figure 2. The mean serine value in the untreated subjects is higher at both three and four hours than the highest mean serine value in the normal persons. On the other hand, the mean value for glycine in untreated subjects does not reach normal mean values at any time during the four hour period. While the serine changes are relatively small (and drawn to a different scale) they are well within the sensitivity limits of the method, and are statistically significant. Comparison of the peak serine values of normal and untreated subjects by the 't' test indicates significance at the 1 per cent level; comparison of the four hour serine values of normal persons with those of untreated subjects yields significance at the 0.1 per cent level.

The total amino acid nitrogen values in the same subjects are shown in Figure 3, the results being generally similar to the curves obtained with the glycine determination.

Figures 4, 5, and 6 illustrate total amino acid nitrogen and the increases above the fasting amino acid nitrogen attributable to glycine and to glycine plus serine. All values were converted to mg. of amino acid nitrogen per 100 ml. plasma. Glycine makes up about 60 per cent of the increase during the first two hours in the normal subjects, and serine accounts for another 3 or 4 per cent. The remainder is presumably due to other amino acids derived from glycine. The patterns suggest a relationship between increases in glycine nitrogen and total amino acid nitrogen, confirming the validity of using total amino acid nitrogen as an index of glycine absorption.

The results of intravenous glycine administration are shown in Figure 7. At the end of five minutes less than 100 micrograms per ml. of plasma remained in each subject of the injected dose, which was calculated to produce a concentration of 500 micrograms per ml. if immediately injected. Thus, over 80 per cent of the injected dose disappeared from the plasma of all subjects within 5 minutes after the injection ended, or 15 minutes from the beginning. Two patients with sprue in relapse displayed clearance rates somewhat slower than the controls. Although the group is small the observations tend to rule out *rapid* clearance as a factor causing the lower plasma concentrations which were found after oral administration of glycine.

TABLE III

Total amino acid nitrogen concentration in plasma after oral administration of 25 Gm. glycine (mg. amino acid nitrogen per 100 ml. plasma)

	Nine normal subjects			Nine treated sprue subjects			Eight untreated sprue subjects		
	Mean	S.D.	Corrected	Mean	S.D.	Corrected	Mean	S.D.	Corrected
Fasting	6.1	0.7	6.1	6.1	0.8	6.1	6.7	1.2	6.7
1 hr.	12.6	2.2	13.1	13.4	2.7	11.6	9.6	1.9	9.4
1.5 hrs.	12.9	1.8	13.4	15.0	3.5	12.8	11.0	2.3	10.7
2 hrs.	11.4	1.4	11.8	15.2	3.9	12.9	11.9	2.4	11.6
3 hrs.	8.2	1.0	8.4	13.1	2.9	11.4	12.1	2.3	11.8
4 hrs.	7.2	0.5	7.3	10.4	2.6	9.3	12.5	2.4	12.2

DISCUSSION

Absorption tests based on the appearance in blood of orally administered substances require knowledge of the metabolic fates and removal rates of the compound under study. Materials which fluctuate as a result of several variables are not well suited to tests of intestinal absorption. For example, blood sugar curves are affected by glycogen stores in the liver, stress, activity, renal excretion, thyroid function, and pancreatic islet cell function in addition to absorption. These

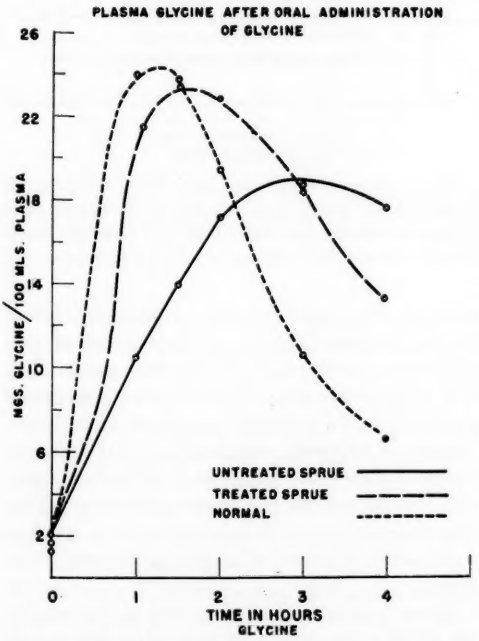

FIG. 1. MEAN PLASMA GLYCINE VALUES AFTER ORAL ADMINISTRATION OF GLYCINE, EXPRESSED AS MG. OF GLYCINE PER 100 ML. OF PLASMA

considerations led Althausen (15) to point out the desirability of using material which is metabolized at a constant rate. Until tracer techniques become more widely applicable to human study, it will probably be necessary to continue utilizing chemical methods. Even with the use of isotopic labels a thorough understanding of chemical pathways is essential.

Plasma glycine normally varies between a rather narrow range, so that increases observed after oral administration are predominantly the result

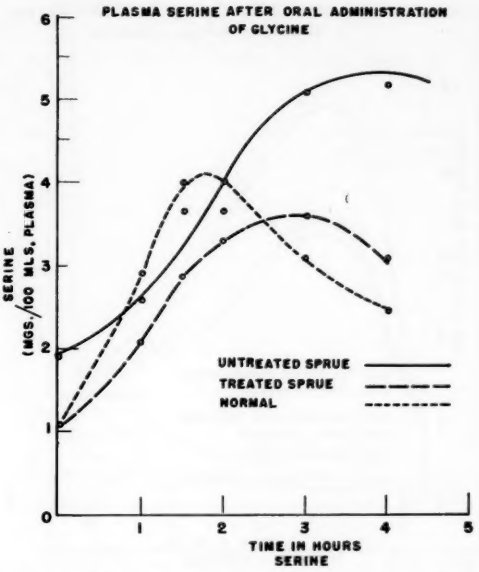

FIG. 2. MEAN PLASMA SERINE VALUES AFTER ORAL ADMINISTRATION OF GLYCINE, EXPRESSED AS MG. OF SERINE PER 100 ML. OF PLASMA

of absorption. In the present study the normal fasting glycine value ranged from 1.05 to 1.73 mg. per 100 ml. of plasma (mean, 1.34; S.D., ± 0.21).

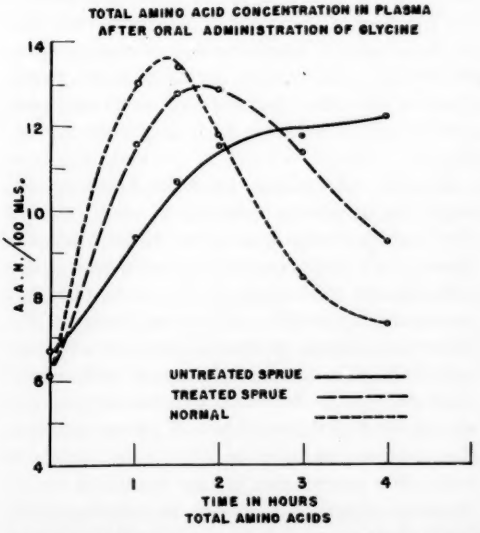

FIG. 3. MEAN TOTAL AMINO ACID VALUES AFTER ORAL ADMINISTRATION OF GLYCINE, EXPRESSED IN MG. OF AMINO ACID NITROGEN PER 100 ML. OF PLASMA

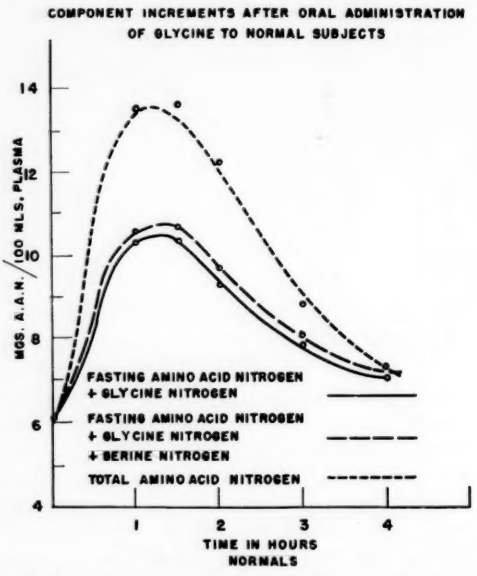

FIG. 4. CURVES SHOWING THE MEAN VALUES OF NINE NORMAL SUBJECTS FOR TOTAL AMINO ACID NITROGEN AND THE INCREMENTS DUE TO GLYCINE NITROGEN AND SERINE NITROGEN

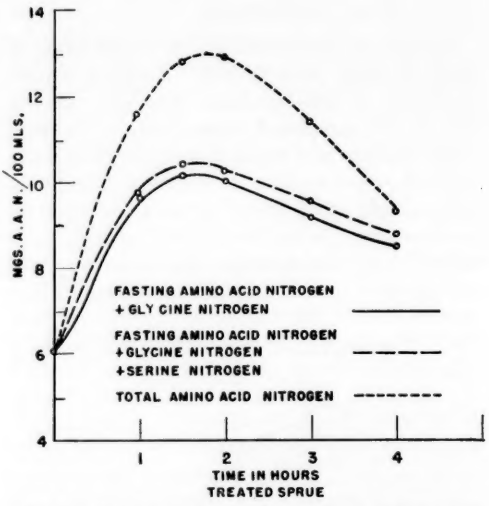

FIG. 5. CURVES SHOWING THE MEAN VALUES OF NINE TREATED SPRUE SUBJECTS FOR TOTAL AMINO ACID NITROGEN AND THE INCREMENTS DUE TO GLYCINE NITROGEN AND SERINE NITROGEN

Stein and Moore (14) reported a range of 1.52 to 1.73 mg. per 100 ml. of plasma in five normal subjects. These values agree with other reports in the literature. The mean value during the peak of absorption is approximately seventeen times the fasting value which is significantly greater than the increases observed with other test compounds currently in use, such as glucose or vitamin A.

Efficient mechanisms exist for restoring elevated plasma glycine values to normal. Among these are pathways leading to the formation of serine (16), threonine (17), methionine (18), ethanolamine (18), choline (18), heme (19), nucleic acid purines (20), and various proteins (21). Nine molecules of glycine are thought to be required for the synthesis of each molecule of porphyrin (22). In addition to the evidence previously cited of the rapid rate of plasma clearance after intravenous injection (4), there is evidence from other sources that glycine is utilized rapidly from the plasma. Edmonds and LePage (20) found that radioactivity from tagged glycine begins to appear in nucleic acids of rats within 20 minutes after administration. Junquiera, Hirsch, and Rothschild (21) report appearance of protein bound radioactivity in the pancreatic secretion of rats beginning one hour after injection of glycine-C^{14} and reaching a peak in three hours. Dimant, Landsberg, and London (23) have reported a rapid incorporation of C^{14}-labelled glycine into the red cell glutathione of humans. They cite evidence that this glycine is in a dynamic state with a half-time value of approximately four days.

There is evidence from a variety of sources (16, 18, 24–27) that folic acid derivatives are needed for the interconversion of glycine and serine. Alexander and Greenberg (24), using an enzyme *in vitro*, found that the reaction predominantly proceeds toward the formation of serine. Pyridoxine is also needed for this reaction (24, 28). Elwyn and Sprinson (26) found that in folic acid-deficient rats conversion of serine to glycine occurred at about one-sixth of the normal rate.

Urinary excretion does not appear to be a major factor in lowering the plasma concentration of glycine. Doolan, Harper, Hutchin, and Shreeve (29) found efficient tubular reabsorption of glycine, with urinary excretion of less than 4

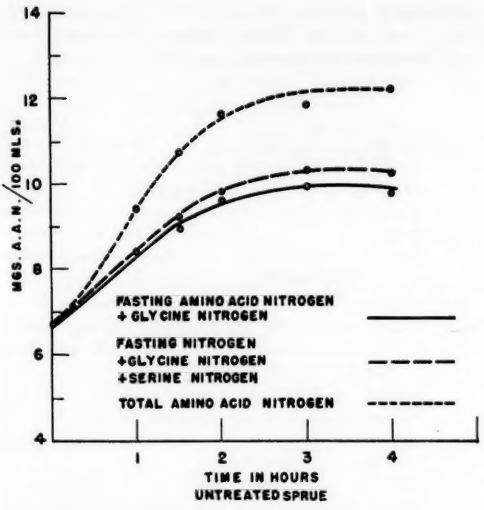

FIG. 6. CURVES SHOWING THE MEAN VALUES OF EIGHT UNTREATED SPRUE SUBJECTS FOR TOTAL AMINO ACID NITROGEN AND THE INCREMENTS DUE TO GLYCINE NITROGEN AND SERINE NITROGEN

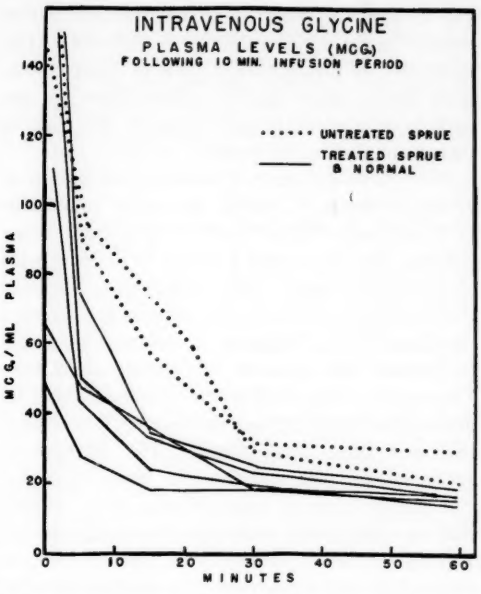

FIG. 7. PLASMA GLYCINE VALUES FOLLOWING INTRAVENOUS ADMINISTRATION OF GLYCINE

per cent even after amino acid loading. In one of our normal subjects the urinary excretion of glycine in four hours after a 25 Gm. oral dose amounted to only 0.54 Gm. or 2.1 per cent.

At the beginning of the present investigation it was thought that patients with sprue in relapse, who are presumed to have a deficiency of folic acid, would show impaired utilization of glycine and altered glycine-serine relationships. However, the curves (Figures 1 through 3) indicate that in general the serine and total amino acid nitrogen values reflect whatever pattern is found with glycine.

The curve of mean total amino acid nitrogen from untreated subjects (Figure 3) is similar to the results presented by Althausen and co-workers (1), who also found the peak of absorption at the fourth hour in a man who retained only 18 to 24 inches of small intestine after extensive surgical resection. These authors found a peak absorption value in normals at one to two hours comparable to results in our series. In other words, the curve of glycine absorption in untreated sprue is like the pattern seen in malabsorption due to anatomical loss of a large portion of the small intestine.

The shape of the various curves appears to be of some diagnostic value. For example, three of the eight untreated subjects achieved a peak glycine concentration as high as the mean peak value for normal controls, but in each instance the peak was delayed in patients with sprue to two, three, and four hours, respectively. In all cases there was a slow continued rise rather than a sharp peak followed by a decline. This pattern was not observed in normal subjects. Curves from treated patients are consistently intermediate between those of patients in relapse and normal subjects. This finding is no doubt due to individual variation in degree of response to therapy. It is felt that the pattern of uptake is of more diagnostic value than absolute concentrations at any given time. Since hastened removal of glycine from blood does not appear to be a factor (Figure 7), delayed intestinal absorption is the most likely explanation for the abnormal curves found in sprue patients. Reduced intestinal motility could be a contributory factor.

Examination of the curves of component increments indicates that glycine accounts for 50 to 60 per cent of the increase in amino acid nitro-

gen at the high point of each curve, and serine constitutes an additional 3 or 4 per cent. The remainder is presumably a pool of other amino acids derived from glycine. There does not appear to be a constant ratio between the concentrations of glycine and serine.

There is no evidence of impaired conversion of glycine to serine. Actually the serine values rise to significantly higher levels in untreated sprue patients than in normal persons or patients with sprue in remission. This observation suggests that although untreated sprue patients are deficient in folic acid the deficiency is not severe enough to impede this pathway of glycine utilization. Presumably suboptimal levels of folic acid and its derivatives affect certain tissues and biochemical reactions more than others. The increase in serine in untreated subjects four hours after glycine dosage remains unexplained. It is possible that an abnormality exists in other pathways involving serine utilization, or that glycine is shunted into the serine pool from another reaction.

SUMMARY

1. Concomitant chemical determinations of plasma glycine, serine, and total amino acid nitrogen after oral glycine administration have been carried out in nine normal subjects, eight persons with sprue in relapse, and nine persons with sprue in remission.

2. The curves indicate a slow rate of intestinal absorption and a delayed peak value in untreated subjects as compared with normals. Treated subjects were intermediate between the two.

3. The curves are similar in pattern for each substance in any given category of subjects, suggesting that serine and total amino acid values are directly related to the rate of glycine absorption from the intestine.

4. There is no evidence of impaired conversion of glycine to serine in patients with sprue in relapse. Such patients display significantly higher concentrations of plasma serine after oral ingestion of glycine than do normal persons, suggesting an impairment of serine utilization.

ACKNOWLEDGMENTS

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STUDIES OF VISCERAL PAIN: MEASUREMENTS OF STIMULUS INTENSITY AND DURATION ASSOCIATED WITH THE ONSET OF PAIN IN ESOPHAGUS, ILEUM AND COLON

By MARTIN LIPKIN [1] AND MARVIN H. SLEISENGER

(From the Department of Medicine, New York Hospital-Cornell Medical Center, New York, N. Y.)

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Past studies of visceral pain have generally been concerned with qualitative aspects of pain production. However, the occurrence of visceral pain has not been studied in terms as quantitative as those used to describe the onset of cutaneous pain. As a result, physiologic concepts of visceral pain which might be based on such measurements have not been derived.

In previous experiments, pain has been produced in the gastrointestinal tract by the application of a distending force to the hollow viscus. A commonly used method of making such studies is that of placing in the viscus a balloon attached to a manometer. In previous studies the magnitude of applied pressure has been identified with the intensity of stimulus required to produce pain. A great variability has been found in the stimulus intensity which resulted in pain in the same and different subjects under the experimental conditions used (1–3). However, there has been no study involving simultaneous measurements of stimulus intensity and duration associated with the onset of pain, as performed in the present experiments.

The objectives of the present investigation were: 1) to produce pain in the gastrointestinal tract with a stimulus, certain characteristics of which could be accurately determined and controlled; 2) to study the relationship between intensity and duration of stimulus required to produce pain; and 3) to assess the reproducibility of these measurements in the same and in different individuals.

METHODS

The apparatus used in these experiments is shown diagramatically in Figure 1. Chamber A was a cylindrical water tank 45 centimeters in diameter, which had a cross-sectional area of 1,590 cm.^{2} at the fluid surface. By means of firm plastic tubing, chamber A was connected to a latex balloon, C. Chamber B was an additional water reservoir, and D and E were mechanically operated valves. Alternate connection of balloon and chambers A or B could be effected by means of the valves. During an experimental determination, the balloon and chamber A were connected. Because of the large cross-sectional area at the fluid surface in chamber A, the height of water did not measurably change. The diameter of the tubing was 0.75 cm., and the entire length of tubing was 290 cm. With the fluid surface in chamber A 100 cm. above the end of the tubing, water flowed from the tubing at a rate of 49 ml. per second. The resistance of the balloons to distention was also studied *in vitro*. Measurements were made of pressures within the balloons at various levels of distention. The maximum pressure attained from expansion of the balloons *in vitro* was 11 cm. of water. In the present experiments, the intensity of the stimulus was measured as the height of water in chamber A. The relationship between the pressure, P_o, determined by the height of water in chamber A, and the pressure, P, applied to the viscus, may be given by equation (I) in which

$$P = P_0 - f_1\left(\frac{\pi}{2} Dl \frac{dD}{dt}\right) - f_2(D) \qquad (I).$$

The function f_1 describes the pressure decline in the tubing, and is equal to the rate of volume expansion of the balloon, where D and l are respectively the diameter and length of the balloon. The function f_2 describes the small pressure differential needed to expand the balloon. In this series of experiments relationships between the intensity of stimulus, P_o, and the time to onset of pain were studied. Relationships between f_1 and f_2 and the onset of pain were not studied. The principle utilized in these studies of visceral pain, in which the externally applied stimulus was maintained constant during an experimental determination, was the same as that used in studies of cutaneous pain (4, 5).

Identical water tanks, valves and tubing were used for all experiments, with a new condom balloon of the same brand in each experiment. Forty-five male and six female subjects ranging in age from 20 to 53 years were studied. Five of the subjects were normal physicians and medical students, and 46 were hospital patients without evidence of gastrointestinal disease or functional

[1] Formerly, United States Pubic Health Service Postdoctorate Research Fellow.

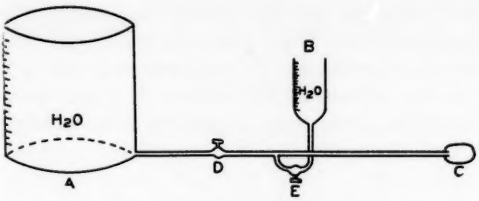

FIG. 1. DIAGRAM OF APPARATUS USED IN THESE EXPERIMENTS

Chamber A was a large water tank, connected by firm tubing to a latex balloon, C. Chamber B was an additional water reservoir; D and E were mechanically operated valves by means of which alternate connection of the balloon and chamber A or B could be made.

disorder. The orientation of the subjects to the experimental procedure was similar to that utilized in studies of cutaneous pain (4, 5). Each subject was carefully instructed regarding the purpose and method of the study. The subjects were asked to signal at the onset of a painful sensation, and then were asked to describe it in terms of quality, position, intensity, duration and radiation. The pain threshold was defined as the time of onset of the earliest perceptible sensation of pain. The description of the sensation given by each subject was carefully noted, and in all instances closely corresponded to classic descriptions of visceral pain (6). Several studies of the colon were performed with the authors as subjects, and in these instances the onset of pain could be clearly defined within the time range noted by the other subjects at the various pressures.

In each experiment, a balloon was attached to the distal end of the tubing. The tube and balloon were then filled with water. With experience at this procedure, it was possible to manipulate the tube and balloon so that air bubbles were eliminated. The tube and balloon were also sufficiently transparent so that small air bubbles could be seen if present and removed. A clamp was applied to the proximal end of the tube to prevent the loss of water from the tube and introduction of air bubbles during intubation. The tube and balloon were then introduced to the desired position. In experiments on the esophagus, the subject swallowed the tube aided by the sipping of iced water, and the tube and balloon were positioned so that the tip was just above the cardio-esophageal junction. Studies of the small intestine were performed in patients who had ileostomies for ulcerative colitis. All patients in this category had been operated on more than two years prior to this procedure and had no symptoms or signs of disease of the ileum. Intubation was performed without any prior preparation. The tube and balloon were introduced into the stoma, and passed 20 cm. For studies of the sigmoid colon, patients were prepared with a low Fleet® enema (hypertonic phosphate solution). A proctoscope was passed to 12 to 15 cm., the tube and balloon inserted, and the proctoscope

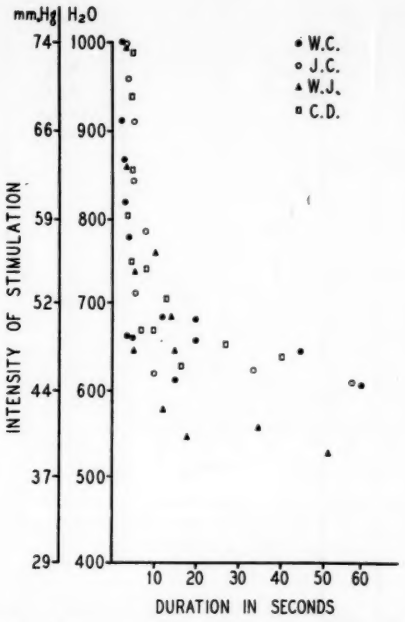

FIG. 2. RELATIONSHIP OF STIMULUS INTENSITY AND DURATION REQUIRED TO ELICIT PAIN, FOLLOWING DISTENTION OF ESOPHAGUS

The intensity of stimulus is shown in mm. of Hg and mm. of H_2O.

removed. The tube and balloon were then passed gradually to 30 to 35 cm. The subjects were supine during all experimental determinations. The position of the tube was determined fluoroscopically with the aid of radiopaque thread tied to the end of the tube.

When the tube and balloon were in the desired position, the clamp was removed and the entire water filled system connected, again making sure to avoid the introduction of air bubbles. Initially, the balloon and distal tubing were connected to the additional water reservoir (chamber B, Figure 1). The height of water in chamber B was set at the same height as the anterior abdominal wall. After an interval of 15 minutes, chamber B was disconnected and chamber A suddenly connected to the system. Operation of the valves was not visible to the subjects. At the onset of pain, the subject relayed a signal and the time was noted. Chamber A was immediately disconnected and chamber B connected to the tubing and balloon, resulting in a flow of water from the balloon into chamber B. In all instances, the sensation of pain subsided with the flow of water from the balloon. The outflow of water into chamber B was also measured. After an interval of five minutes, the determination was repeated.

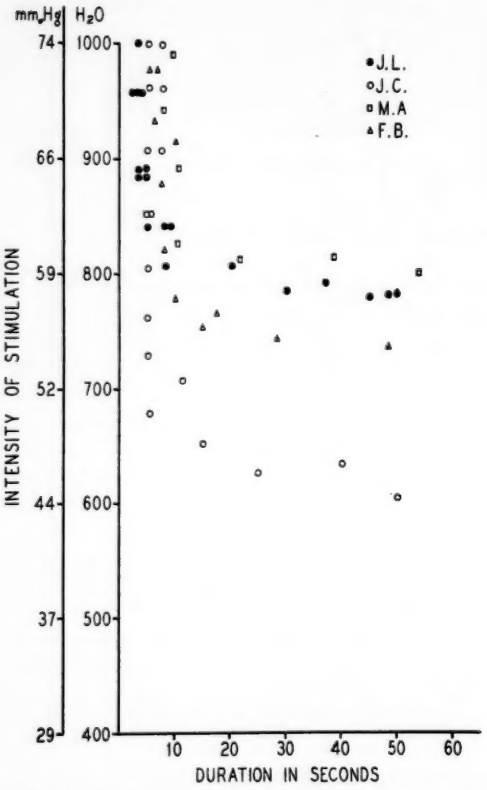

FIG. 3. RELATIONSHIP OF INTENSITY AND DURATION OF STIMULUS REQUIRED TO ELICIT PAIN FOLLOWING DISTENTION OF ILEUM

In the first series of experiments the relationship of the intensity and duration of the stimulus to onset of pain threshold was studied. Four experiments with esophageal intubation were performed on four subjects. Four experiments were performed on four patients with ileostomies, and seven determinations on the sigmoid colon of six subjects. In the last group, four of the subjects were doctors and medical students.

In the second series of experiments, P_o was set at 100 cm. of water, and the duration to pain threshold was studied in the sigmoid colon. The frequency distribution of 171 observations made on 37 subjects was noted. Repeated observations were also made on four subjects over a period of three to four months.

RESULTS

In Figures 2, 3, and 4, the relationship of intensity of stimulation to duration measured for threshold pain originating in the esophagus, ileum and colon are shown. When the intensity of stimulation was large, pain was usually the first sensation perceived. In measurements with low intensity stimulation accompanied by longer duration prior to the onset of pain, the subjects often noted the appearance of a sensation of pressure prior to pain. In all instances, the sensation of pressure was related to a larger area than was pain. From within the area in which pressure was perceived, the sensation of pain arose, and was described as aching in quality. Esophageal pain was referred to the midline at a level of the second to fifth thoracic segments; pain from the lower ileum at a level of the eighth to tenth thoracic segments and colonic pain to the eleventh and twelfth thoracic segments within 3 inches to the right and 3 inches to the left of the midline. In Figures 2 through 4 it is seen that an increase in intensity of stimulation is accompanied by a decrease in duration required for the response. In addition, greater variation in duration is noted at lower intensities of stimulation than at higher intensities. The minimum time to pain threshold was 2 seconds for the esophagus, 3 seconds for the ileum, and 2 seconds for the colon. In experiments on the esophagus, if no pain appeared within 40 to 60 seconds after the application of low intensity stimulation, none appeared thereafter. The corresponding durations were 50 to 55 seconds for ileal measurements, and 40 to 60 seconds for the colon. The minimal intensity of stimulation required to elicit esophageal pain was 520 to 620 mm. H_2O. The corresponding intensity for experiments on the ileum was 600 mm. H_2O for subject J. C. However, the minimal intensity required for the other subjects was higher, at 740 to 800 mm. H_2O. In experiments on the colon, the minimal intensity was 540 to 670 mm. H_2O with greater variability at lower intensities of stimulation than was present in determinations on esophagus and ileum.

In the second series of determinations, the duration to pain threshold was studied in the sigmoid colon of 37 subjects at a single intensity of applied stimulation. Figure 5 shows the total compilation of these pain threshold measurements. The mean pain threshold value was 6.6 seconds, with a standard deviation of $\pm$ 3.6 seconds. The results of repeated studies made on four subjects are shown in Figure 6. The time thresholds

measured for these subjects show little variation when repeated at intervals of several weeks.

DISCUSSION

Characteristics of stimulus

Studies of pain sensation carried out in the past have stressed the importance of understanding many characteristics concerned with evaluation of stimulus and response (4). Certain principles derived from this past experience have been followed in the present study. In order to evaluate adequately the occurrence of the sensation of pain, the stimulus should meet the following requirements:

The stimulus used under experimental conditions should be very similar to the noxious stimulus which causes naturally occurring pain. The characteristics of the stimulus should be measured, and should be controlled to a fine degree. It should, therefore, be possible to maintain the externally applied stimulus constant over a given duration and to reproduce easily any stimulus intensity. The stimulus should produce the sensation of pain in such a manner that the sensation is clearly identified.

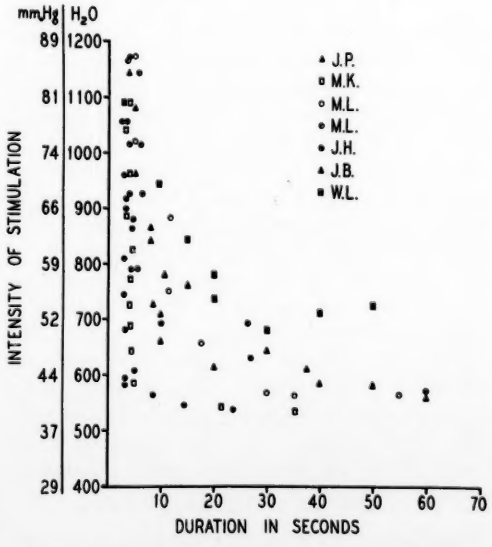

FIG. 4. RELATIONSHIP OF INTENSITY AND DURATION OF STIMULUS REQUIRED TO ELICIT PAIN, FOLLOWING DISTENTION OF SIGMOID COLON

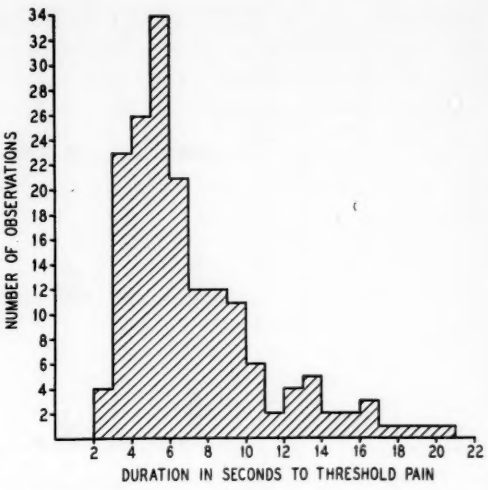

FIG. 5. FREQUENCY DISTRIBUTION OF DURATIONS TO PAIN THRESHOLD FOLLOWING DISTENTION OF SIGMOID COLON, AT A SINGLE INTENSITY OF APPLIED STIMULATION

Intensity-duration measurements

Studies of the responses of a wide variety of living tissues to many types of stimulation have revealed a characteristic relationship between the intensity of the stimulus and the duration required to produce a given response, namely, that a greater intensity of stimulation is associated with a shorter duration required to elicit the response (7). The same relation of stimulus intensity and duration has been shown in the production of cutaneous pain (8). The fact that the onset of visceral pain appears to follow the same general rule with respect to stimulus intensity and duration is of particular interest. Intensity-duration curves have not previously been demonstrated for the onset of visceral pain.

As in other studies of intensity-duration curves (4, 9), the term "rheobase" may be used to denote the intensity of stimulation below which the response will not occur. The term "utilization time" may be used to denote the minimum time of stimulation effective for rheobase intensity. In these experiments, the rheobase for 3 subjects was in a higher range (740 to 800 mm. H_2O) than for the other 11 subjects (520 to 700 mm. H_2O). The values for higher rheobase occurred following distention of ileum in the group with previous ul-

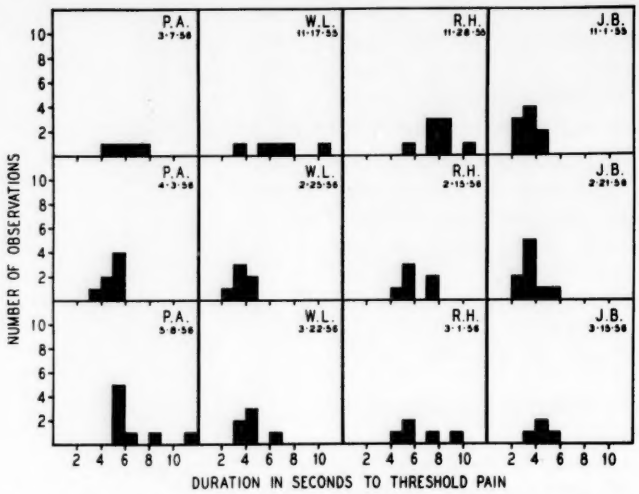

FIG. 6. DURATION TO THRESHOLD PAIN FOLLOWING DISTENTION OF SIGMOID COLON, AT A SINGLE INTENSITY OF STIMULATION

Repeated observations made on four subjects. P_0 was set at 100 cm. of water. The duration to pain threshold for each subject is shown on the abscissa. The number of observations for each of the three experiments performed on each subject is shown on the ordinate.

cerative colitis and colonic resection. Differences in rheobase values should reflect variations in the individual tissues, since the apparatus was the same in all determinations. The differences in rheobase might be caused by variations in innervation of the segments of a viscus, or by variations in resistance to distention present in the viscus. In addition, different balloon lengths present after distention in segments of varying resistances could have resulted in stimulation of unequal lengths of intestine. The relationship of the rate of distention of the viscus to the onset of pain cannot be stated with certainty. The possibility that a lower rheobase is associated with an increase in the rate of distention of the viscus is supported by the observation that a greater intensity of stimulation causes an increased rate of expansion of the balloon *in vitro*, and an earlier onset of pain *in vivo*. An earlier onset of pain at a given stimulus intensity would result in a lower rheobase. However, increased intramural tension in the viscus associated with a decreased rate of distention might also result in an earlier onset of pain and a lower rheobase.

There are certain corrections which should be made to the externally applied pressure measurements in order to evaluate the actual pressure applied to the wall of the intestine. These corrections relate to the pressure drop in the tubing, occurring when large rates of flow exist, and to the pressure differential associated with the elastic expansion of the balloon as described in equation (I). If the actual pressure applied to the viscus were known, then a more accurate evaluation of intensity-duration relationships would be possible. However, in the present preliminary study, as in studies of cutaneous pain (5, 8), the intensity of the externally applied stimulus was measured, rather than measurement of the actual stimulus applied to the nerve endings. It is believed that despite these omissions, the intensity-duration relationship noted is correct. Thus, a correction for pressure drop would not be expected to decrease the vertical spread of the data; a general downward shift of the points shown would increase the ratio of maximum to minimum pressure.

Comparisons of visceral and cutaneous pain

In studies of tissue response to stimulation, it has been noted that a tissue may react to a stimu-

lus in such a way as to oppose alteration of the normal status of the tissue (7, 10). This characteristic which has been termed "accommodation" or "adaptation" may be a significant part of the response to low intensity stimulation. It has also been noted in studies of the response of cutaneous tissue to thermal stimulation utilized to elicit pain. In the latter case, it has been shown that when normal skin is exposed to thermal radiation, cutaneous blood flow to the area is increased. The thermal energy available to induce a rise in skin temperature is thus reduced, and an abnormal rise in skin temperature is opposed (11). The occurrence of cutaneous pain is related to an inability of increased blood flow to prevent a temperature rise, and if the skin temperature rises to 45° C., pain occurs. The onset of pain in this instance has been shown to be related to the onset of tissue damage and is believed to be caused by protein denaturation along the nerve endings (12).

In studies of cutaneous pain, it has been possible to study the reproducibility of the time threshold to pain, and at the same time to evaluate the response of accommodation to the stimulus. It has thus been shown that with high intensity stimulation cutaneous blood flow does not change, and the variation in time threshold is very small. With low intensity stimulation, however, cutaneous blood flow does increase, and the time threshold is more variable (11).

Studies relating the onset of visceral pain to stimulus intensity and to local tissue changes have thus far not been carried out as precisely as the manner in which cutaneous pain has been studied. However, on the basis of observations made here and in other studies, certain similarities regarding the production of visceral and cutaneous pain may be noted. Lower intensities of stimulation in both instances are associated with greater variation in the time threshold for pain. It is also known that lower intensities of stimulation permit the tissues to undergo various changes of accommodation or adaptation before the onset of pain, which high intensities of stimulation do not. Thus, after the application of pressure to a viscus, great increase in diameter of the viscus may occur (1). This may involve changes in length and tension of smooth muscle fibers (13). In the occurrence of visceral pain, it may be postulated that differences in accommodation result in greater variation in the time threshold for pain than occurs with cutaneous pain. In addition, the fact that pain is more likely and accommodation less likely to occur with high intensity stimulation, makes it appear that changes of accommodation act to oppose the onset of pain. It would seem that the changes which take place in the viscus in response to pressure might function in the same manner as changes in cutaneous blood flow after thermal stimulation. That is, they would prevent the stimulus from causing the onset of pain. In the case of thermal cutaneous stimulation, the onset of pain is also associated with the production of tissue damage. Whether the onset of visceral pain is also associated with the onset of tissue damage is not known at present.

SUMMARY

The relationship between the intensity and duration of stimulus required to produce pain in the esophagus, ileum and colon has been studied in 51 subjects. Intubation of the organ was performed with balloon and catheter. The balloon-catheter system was attached to a water chamber characterized by a large cross-sectional area at the fluid surface. The intensity of the stimulus was measured as the height of water in the chamber, and the height of water did not measurably change during each experimental determination. The duration to the earliest perceptible sensation of pain was measured.

A relationship of stimulus intensity and duration was found for the onset of visceral pain, which is similar to that shown for cutaneous pain. Intensity-duration curves plotted for studies of the three organs were very similar. Differences in rheobase values were believed to be due to properties intrinsic in the tissues and to variations in the rate of distention of the segment of viscus.

The mean duration to colonic pain threshold in 37 subjects studied at a single stimulus intensity was 6.6 seconds with a standard deviation of ± 3.6 seconds. Repeated observations made on four subjects with several weeks between experiments revealed little variation in pain threshold for each subject.

Comparison of the characteristics of intensity-duration curves obtained from measurements of visceral and cutaneous pain revealed the possi-

bility that changes of local tissue accommodation prevent the onset of both varieties of pain.

ACKNOWLEDGMENTS

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THE EFFECTS OF ACUTELY INCREASED SYSTEMIC RESISTANCE ON THE LEFT ATRIAL PRESSURE PULSE: A METHOD FOR THE CLINICAL DETECTION OF MITRAL INSUFFICIENCY

By EUGENE BRAUNWALD, G. H. WELCH, JR., AND ANDREW G. MORROW

(From the Laboratory of Cardiovascular Physiology and the Clinic of Surgery, National Heart Institute, Bethesda, Md.)

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In 1921, Wiggers and Feil in a study of experimental mitral insufficiency observed an elevation of left atrial pressure when the aorta was constricted (1). This finding has subsequently been confirmed both in experimental animals (2, 3) and in a circulatory model (4). In recent experiments "mitral" regurgitant flow was produced and measured by permitting blood to pass from the left ventricle through a flow meter into the left atrium (5). It was observed that with any given mitral regurgitant orifice the regurgitant flow could be substantially increased by elevating the resistance to left ventricular ejection with graded aortic constriction.

The experimental observations listed above suggested that acutely increased resistance to left ventricular ejection in patients with mitral regurgitation should also result in an elevation of left atrial pressure. It was further reasoned that such elevations of left atrial pressure would not occur in patients without mitral valve disease or with pure mitral stenosis and that this difference in response might therefore be of value in the clinical detection of mitral regurgitation. In addition to such observations in patients, it was considered desirable to determine the effects of elevated aortic pressure on the left atrial pressure pulse in animals before and after the production of experimental mitral regurgitation.

EXPERIMENTAL OBSERVATIONS

Methods. Dogs weighing 14.5 to 20.4 Kg. were studied with intravenous pentobarbital anesthesia and positive pressure respiration. The left chest was entered through the fifth intercostal space. A perforated tygon tube was placed into the left atrium through the appendage; by advancing the end of the tube through the mitral valve into the left ventricle mitral regurgitation was induced and could be abolished by withdrawal of the tube (Figure 1). Resistance to left ventricular ejection was increased either by an infusion of norepinephrine (0.008 mg. per ml. isotonic saline), or by means of graded constriction of the ascending aorta with an aneurysm clamp. Pressures were recorded from the left atrium and from the aorta proximal to the constriction. In two experiments a Potter turbine flow meter (6) was introduced into the aorta for the continuous registration of systemic flow. All recordings were made on a multichannel oscillograph.

RESULTS

Results representative of 26 experiments performed in eight dogs are illustrated in the experiment shown in Figure 2A. In the absence of mitral regurgitation, striking elevations of aortic pressure raised the v point of the left atrial tracing relatively little. A degree of mitral insufficiency which produced only slight elevation of the v point was then induced. A striking elevation of the left atrial v point occurred when aortic pressure was then raised by aortic constriction. This finding was consistent regardless of whether aortic pressure was elevated by aortic constriction or by norepinephrine infusion.

In two experiments systemic flow was recorded while norepinephrine was infused. A greater depression of systemic flow and greater elevation of left atrial mean and v point pressures occurred when mitral regurgitation was present than when absent.

CLINICAL OBSERVATIONS

Selection of patients and methods. A total of 20 patients has been studied. Group I consists of seven patients without mitral insufficiency, of whom six had "pure" mitral stenosis on the basis of clinical findings, the demonstration at left heart catheterization of a diastolic pressure gradient between left atrium and left ventricle, and by palpation of the valve at operation. The seventh patient in this group, J. T., had an atrial septal defect without associated mitral insufficiency; this diagnosis

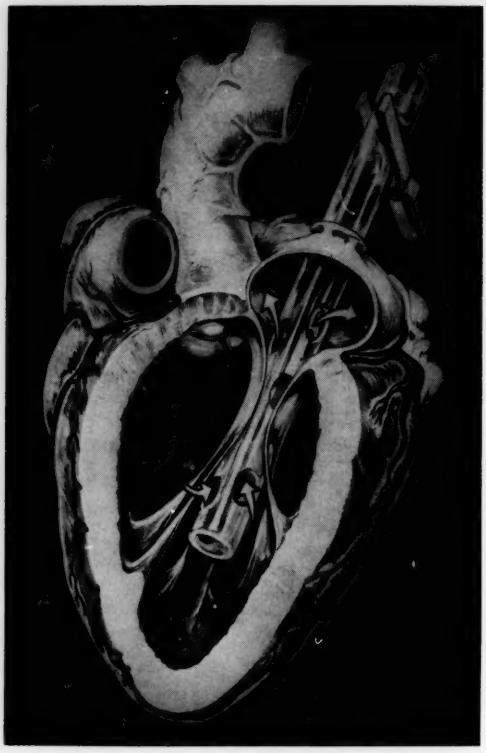

FIG. 1. DIAGRAM OF THE EXPERIMENTAL METHOD OF PRODUCTION OF REVERSIBLE MITRAL INSUFFIENCY

was confirmed at operation. Group II consists of thirteen patients with mitral insufficiency. The six patients in Subgroup IIA had mitral stenosis proved by the presence of a diastolic pressure gradient between left atrium and left ventricle and by operative findings, but in all of these patients a definite regurgitant jet was also palpable. The seven patients in Subgroup IIB presented the clinical picture of "pure" mitral insufficiency; the presence of a left to right shunt was excluded by right heart catheterization.

Left atrial pressures were measured by the technique of transbronchial left heart catheterization, which has been described in detail (7). In the first eight patients norepinephrine was infused while the bronchoscope was in place and left atrial pressure was recorded through the transbronchial needle. In the other twelve patients, following measurement of left ventricular pressure with a polyethylene catheter threaded through the transbronchial needle, both the bronchoscope and needle were removed and the catheter was left *in situ*. After a 20 to 30 minute period during which the patient returned to a more basal state, the catheter was withdrawn into the left atrium and the norepinephrine then infused.

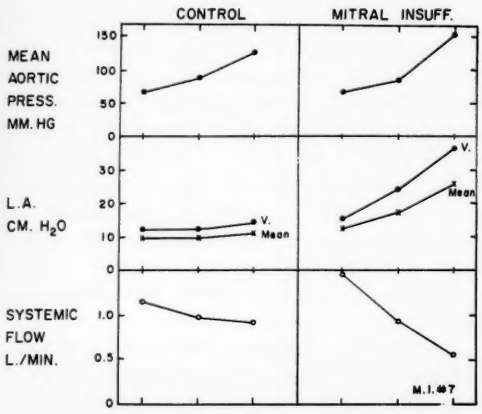

FIG. 2B. ALTERATIONS IN FORWARD SYSTEMIC FLOW ACCOMPANYING ELEVATION OF AORTIC PRESSURE BEFORE AND AFTER THE PRODUCTION OF MITRAL INSUFFICIENCY

Aortic pressure elevated by norepinephrine infusion.

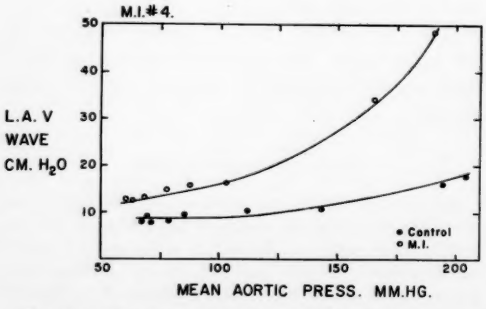

FIG. 2A. RELATION BETWEEN LEFT ATRIAL v POINT PRESSURE AND MEAN AORTIC PRESSURE IN DOGS BEFORE AND AFTER THE PRODUCTION OF MITRAL INSUFFIENCY

Aortic pressure elevated by aortic constriction.

In the measurement of left atrial pressures, a Statham P23 A strain gauge was employed with the transbronchial needle, and a P23 D gauge with the polyethylene catheter. Left atrial and systemic arterial pressures and the electrocardiogram were continuously recorded and visually monitored on a multichannel photographic recorder.

Following control observations, norepinephrine was infused at approximately 10 to 20 γ per minute. The infusion was continued until systolic arterial pressure became significantly elevated (approximately three to five minutes). The infusion was then stopped and measurements continued until the arterial pressure returned to control levels. Pressure measurements were averaged over two respiratory cycles. Mean pressures were obtained either by electrical or planimetric integration.

TABLE I

Hemodynamic observations before and during norepinephrine infusion

Patient	Before norepinephrine infusion*					During norepinephrine infusion					Elevation L. A. v × 100 / Elevation F. A. syst. (%)
	F. A.		L. A.		Heart rate	F. A.		L. A.		Heart rate	
	s/d	m	v	m		s/d	m	v	m		
Group I—No mitral insufficiency											
D. G.†	114/54	76	34	29	96	156/79	104	36	25	40	5
D. S.†	138/61	93	12	9	64	186/73	113	22	14	50	21
I. W.	80/46	60	13	14	100	166/67	116	15	22	64	2
J. G.	136/68	91	10	10	76	164/75	103	14	13	60	14
B. C.	104/55	78	19	19	80	163/72	100	23	21	68	7
J. B.†	124/66	80	22	19	88	151/58	98	23	19	50	4
J. T.	116/77	88	10	8	68	172/88	120	14	11	52	7
Group IIA—Mitral insufficiency and stenosis											
E. C.†	133/72	91	10	9	104	182/91	122	14	11	68	8
J. M.†	123/49	84	10	5	60	206/78	110	35	21	40	30
G. S.	128/75	91	38	35	92	163/88	110	47	41	100	26
E. P.	151/74	102	33	23	104	200/80	123	58	34	80	51
N. M.†	179/78	109	26	20	58	225/85	132	38	25	68	26
H. C.†	128/64	81	25	16	72	181/79	128	42	23	68	32
Group IIB—Pure mitral insufficiency											
L. G.†	134/77	103	20	13	96	198/105	134	45	26	65	39
J. L.†	98/60	76	21	19	66	128/79	97	36	30	52	50
M. S.†	131/71	92	3	3	96	184/105	126	31	13	72	53
W. B.	127/76	94	12	10	84	187/89	120	35	20	64	38
K. S.	135/69	95	38	17	88	203/91	148	92	42	77	79
W. C.	111/67	87	48	28	96	146/83	102	92	46	92	126
M. T.†	167/91	122	32	24	72	197/104	152	48	32	80	53

* F. A., femoral artery pressure; L. A., left atrial pressure; s, systolic; d, diastolic; m, mean; v, v point pressures. All pressures are in mm. Hg.

† Observations made 20 to 30 minutes following bronchoscopy.

RESULTS

The majority of the patients described a brief sensation of pounding in the chest and head accompanying the norepinephrine infusion. Several patients developed occasional premature ventricular contractions during the infusion and one had a brief period of bigeminal rhythm. No complications resulted from the left heart catheterizations.

The results of the clinical studies are summarized in Table I. In the patients without mitral regurgitation (Group I) only a slight elevation of left atrial pressure occurred as systolic arterial pressure was raised (Figures 3 and 4). The ratio of the elevation of the left atrial v point pressure to the rise in systemic arterial systolic pressures ranged from 2 to 21 per cent, averaging 9 per cent. In contrast, in all but one of the patients with mitral regurgitation (Group II) a substantial rise in left atrial pressure occurred as systemic pressure was elevated (Figures 3 and 5). The ratio of the elevation of the left atrial v point pressure to the rise in systemic arterial systolic pressure ranged from 8 to 126 per cent, averaging 47 per cent. In all but one of these patients (E. C.), this ratio exceeded the highest ratio obtained in patients without mitral insufficiency. The ratios tended to be lower in the patients with combined mitral stenosis and insufficiency (Group IIA) than in the patients with pure mitral insufficiency (Group IIB). In Group IIA the ratios ranged from 8 to 51 per cent, with an average of 30 per cent, while in Group IIB the range was from 38 to 126 per cent, with an average of 63 per cent.

DISCUSSION

When norepinephrine is infused in the absence of mitral insufficiency, left atrial pressure is increased only slightly. The stroke work of the left ventricle is increased by both the elevation of aortic pressure and by the bradycardia. The increase in left ventricular stroke work elevates left ventricular end-diastolic and left atrial pres-

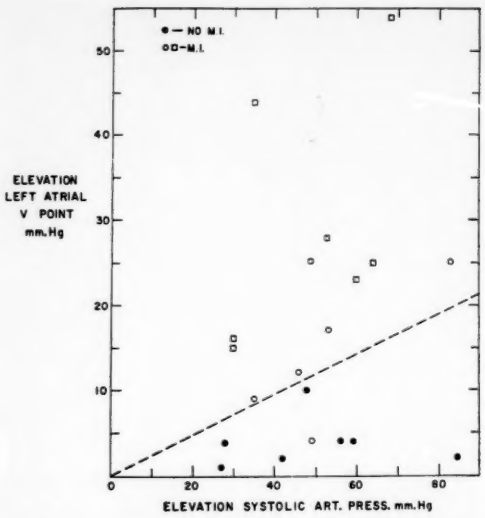

FIG. 3. RELATIONSHIP OF ELEVATION OF LEFT ATRIAL V POINT PRESSURE TO ELEVATION OF FEMORAL ARTERIAL SYSTOLIC PRESSURE IN PATIENTS WITH AND WITHOUT MITRAL INSUFFICIENCY

Closed circles represent patients without mitral insufficiency; open circles, patients with mitral stenosis and insufficiency; open squares, patients with pure mitral insufficiency.

sure (8). However, the elevation of the left ventricular function curve by norepinephrine (9) acts in the opposite direction and tends to decrease left atrial pressure. When mitral insufficiency is present, norepinephrine also increases the systolic pressure gradient across the regurgitant orifice and thereby results in increased mitral regurgitant flow and a substantially greater increase in left atrial pressure. In the dogs in which forward systemic flow was metered (Figure 2b) this elevation of left atrial pressure was accompanied by a fall in forward cardiac output. Other experiments on metered mitral regurgitant flow have shown that total cardiac output, *i.e.*, the sum of regurgitant flow and forward cardiac output, remains relatively constant as forward cardiac output and regurgitant flow vary reciprocally with aortic constriction (5). Thus, in the presence of mitral regurgitation, if left ventricular emptying across the aortic valve is hindered, the blood seeks the other path available to it and mitral regurgitant flow is increased.

It would seem to follow that with any given regurgitant orifice, any mechanism operating to increase the left venrticular systolic pressure, such as systemic hypertension or aortic stenosis, would augment mitral regurgitant flow. Similarly, when ventricular systolic pressure is elevated by aortic insufficiency, coexistent mitral regurgitation is poorly tolerated (10, 11).

Norepinephrine was the agent chosen to increase peripheral resistance acutely because its effects are transient and the arterial pressure level could therefore be precisely controlled by the speed of infusion. Since it is well recognized that this drug increases myocardial contractility as well as peripheral resistance (9, 12), it is likely that

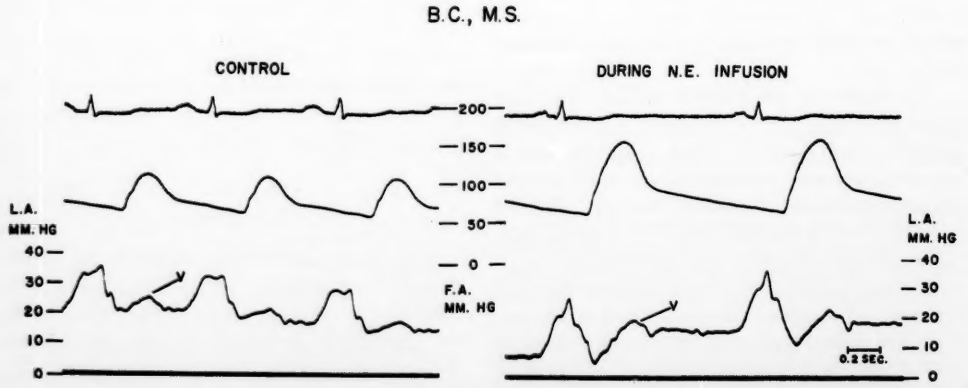

FIG. 4. FEMORAL ARTERIAL (F.A.) AND LEFT ATRIAL (L.A.) PRESSURES BEFORE AND DURING NOREPINEPHRINE (N.E.) INFUSION IN PATIENT B. C. WITH PURE MITRAL STENOSIS

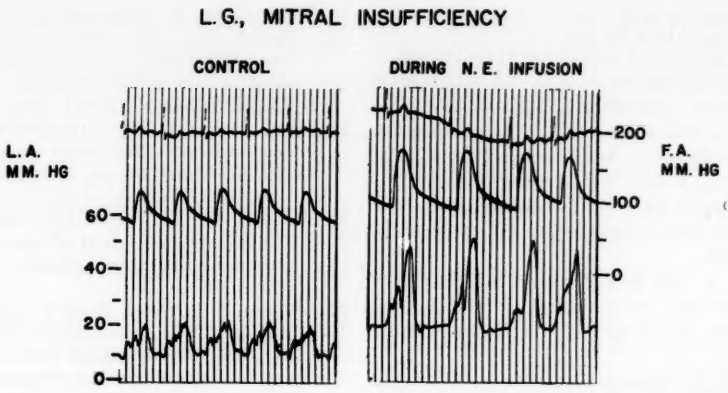

FIG. 5. FEMORAL ARTERIAL AND LEFT ATRIAL PRESSURES IN PATIENT L. G. WITH PURE MITRAL INSUFFICIENCY

Vertical time lines indicate 0.1 second.

if an agent such as methoxamine, which acts predominantly on the periphery, had been employed the elevation of atrial pressure would have been greater in both groups of patients.

The extent to which left atrial pressure is elevated during norepinephrine infusion would appear to be of diagnostic value. The presence of severe, pure mitral insufficiency can generally be recognized in the left atrial pressure tracing (13). When mitral regurgitation is associated with mitral stenosis, the pressure pulse is modified and sometimes is indistinguishable from that obtained in patients with "pure" stenosis. However, in the present study, the elevation of arterial pressure resulted in greater elevation of left atrial pressure in patients with combined mitral stenosis and insufficiency than in patients with "pure" mitral stenosis. Therefore, the technique described affords opportunity for the detection of mitral insufficiency in patients with associated mitral stenosis.

Patients who present apical systolic murmurs without elevation of left atrial pressure may present difficult diagnostic problems. While an elevated left atrial v point may suggest the presence of mitral regurgitation, it should be emphasized that this point is often the highest in the left atrial pressure pulse during the cardiac cycle in normal man (14). The effect on left atrial pressure of increasing resistance to aortic ejection may be of value in establishing or excluding the presence of mitral insufficiency in such patients.

SUMMARY

The effects of increased resistance to ventricular ejection on the left atrial pressure pulse were studied in eight dogs with reversible mitral insufficiency. In the absence of mitral insufficiency, elevated aortic pressure resulted in only slight elevations in the left atrial v point. Similar increases in aortic pressure in the presence of mitral insufficiency resulted in striking elevations of mean and v point pressures.

Utilizing the techniques of transbronchial left heart catheterization and norepinephrine infusion, similar observations were made in 20 patients. In the absence of mitral insufficiency (seven patients) the average ratio of increase of v point pressure to increase in systolic peripheral arterial pressure was only 9 per cent. In contrast, in 13 patients with mitral insufficiency this ratio averaged 47 per cent. Although the highest ratios were observed in patients with "pure" mitral insufficiency, striking increases in left atrial pressure were seen in patients with both mitral stenosis and insufficiency. The physiologic mechanisms involved and the usefulness of this technique in the clinical recognition of mitral insufficiency are discussed.

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A TANDEM FOREARM PLETHYSMOGRAPH FOR STUDY OF ACUTE RESPONSES OF THE PERIPHERAL VEINS OF MAN: THE EFFECT OF ENVIRONMENTAL AND LOCAL TEMPERATURE CHANGE, AND THE EFFECT OF POOLING BLOOD IN THE EXTREMITIES [1]

By J. EDWIN WOOD[2] AND JOHN W. ECKSTEIN[3]

(From the Department of Medicine, Boston University School of Medicine, and the Evans Memorial, Massachusetts Memorial Hospitals, Boston, Mass.)

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The primary mechanism of action of the smooth muscle of the veins upon their contained blood is one of imparting pressure to the blood by a surrounding force. This mode of action is to be distinguished from that of the smooth muscle of the arterioles, which is one of maintenance of arterial pressure through resistance to the outflow of blood. The function of the arterioles as resistive elements in the circulation is evaluated by the relationship between the volume of the flow of blood through the arterioles per unit period of time and the change in pressure across the arterioles. The function of the veins as containers of the blood volume is best evaluated by the relationship between a change of the volume of the veins and the associated change of the pressure of the veins.

It is the purpose of this paper to describe a new method for the simultaneous study of the functions of the arterioles and the veins of the extremities of man. In addition, the effects of several stimuli upon veins and arterioles of the forearm are also described. The stimuli employed were the reduction of the temperature of the room, the reduction of the temperature surrounding the forearm under study, and the congestion of the veins of the three extremities not being studied.

METHODS

A water plethysmograph made of lucite which had two chambers was used in all of the experiments (Figure 1). Each chamber was 8.5 cm. long and had separate sleeves which were thin, loose fitting rubber. The outer end of each sleeve was everted and attached to a flange at its respective end of the plethysmograph. The inner ends of the two sleeves were attached to the permanent plate that separated the two chambers. Thin (3 mm.), rigid plates, held firmly in place, separated the two sleeves in the center of the plethysmograph and came into close apposition with the skin of the forearm. A change of volume in one chamber did not alter the volume of the other chamber. Similar plates also prevented the sleeve from bulging out at the ends of the plethysmograph (1). In effect, the instrument consisted of two short plethysmographs placed in tandem on the forearm.

A pneumatic cuff which was 8 cm. wide was placed on the wrist just beyond the distal end of the plethysmograph. This cuff was inflated to suprasystolic pressure prior to each determination. A pneumatic cuff 12.5 cm. wide was placed on the upper arm several centimeters distant from the proximal end of the plethysmograph. Pressure in this cuff was monitored with a water manometer. Unless stated otherwise, the temperature of the water in the plethysmograph was maintained at 32° C. The total volume of the forearm segment was determined by the displacement of water from the plethysmograph. Change in volume of the forearm resulted in change in the water level of the plethysmograph. This change of water level was detected with two partially immersed electrodes, constructed as described by Cooper and Kerslake (2), used in conjunction with a Sanborn strain gauge amplifier and a Sanborn direct writing instrument.[4] The recording system was calibrated by introducing known quantities of water into the plethysmograph. Venous pressure was measured with a Statham P23D strain gauge from a small polyethylene catheter inserted into a forearm vein (Figure 1). Venous pressure was also recorded with the Sanborn direct writing instrument.

The exact relationships between changes of pressure within the forearm veins and the associated changes of volume of these veins were studied as follows: The subject was seated with his forearm in the plethysmograph at heart level. Change of the volume in the proximal

[1] This study was supported by a grant from the Life Insurance Medical Research Fund.

[2] Massachusetts Heart Association Research Fellow.

[3] Special Research Fellow of the National Heart Institute. Present address: Department of Internal Medicine, University Hospitals, Iowa City, Iowa.

[4] Construction of electrodes and adaptation to Sanborn strain gauge amplifier by Sanborn Company, Waltham, Massachusetts.

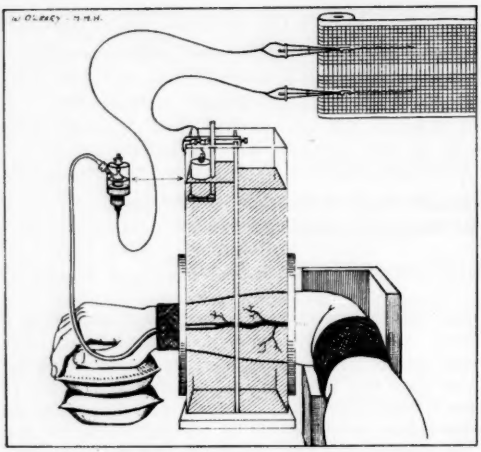

FIG. 1. THE TWO CHAMBER PLETHYSMOGRAPH

Strain gauge, venous catheter and water level electrodes also shown.

chamber of the plethysmograph, change of the volume in the distal chamber of the plethysmograph and pressure in a vein in the distal chamber of the plethysmograph were measured simultaneously. Tracings were obtained with and without water in the proximal chamber of the plethysmograph. The water level in the plethysmograph was such that the external hydrostatic pressure exceeded the original venous pressure at the tip of the catheter (without water in the plethysmograph). The original venous pressure at the tip of the catheter, relative to atmospheric pressure, will be referred to hereafter as *natural local venous pressure*. By placing the membrane of the strain gauge at the level of the water in the distal chamber (Figure 1), the recording obtained was internal venous pressure *minus* the external hydrostatic pressure at the level of the tip of the catheter (referred to hereafter as the *effective venous pressure*). The water level in the proximal chamber was kept 3 mm. lower than that in the distal chamber so that it could not of itself congest the veins of the distal segment. Pressure in the cuff on the upper arm was raised by 1 mm. Hg increments until initial pressure and volume changes had occurred. Following this, pressure in the cuff was raised by 5 mm. Hg increments for a total of 30 mm. Hg.

All additional venous pressure-volume experiments were performed as follows: Water was added to both chambers of the plethysmograph to a level such that the external hydrostatic pressure was certain to exceed the natural local venous pressure of the subject. This water level (3 mm. lower in the proximal chamber of the plethysmograph) was maintained throughout each experiment. Change in volume of the distal segment only was then measured. Pressure within the veins of the forearm in the plethysmograph was not measured. Pressure in the proximal cuff was raised above atmospheric pressure by 1 mm. Hg increments until the first small positive deflection in forearm volume (distal segment) occurred. Sudden release of pressure in the cuff resulted in immediate return of the volume of the forearm to its original level unless the rise in volume was artifactual. The cuff pressure relative to atmospheric pressure which was required to produce this change of forearm volume ranged from 15 to 25 mm. Hg and was referred to as "cuff zero." Cuff pressure was raised further from "cuff zero" by six successive 5 mm. Hg increments. Forearm volume was allowed to reach a new equilibrium prior to initiation of each increment. The small volume increase produced by "cuff zero" itself was included in the first increment. The volume increments were plotted additively against effective venous pressure (Figure 2). The volume increase in ml. per 100 ml. forearm tissue with a rise in effective venous pressure of 30 mm. Hg was called *venous volume* [30] (Figure 2).

The effect of sudden venoconstriction upon the volume of the veins when water was in the plethysmograph as above but without pressure in the proximal cuff was investigated, utilizing the method just described. The subject was studied in the recumbent position with the plethysmograph on one forearm and an intravenous saline infusion in the opposite arm. A venous pressure-volume curve was obtained; then pressure in the proximal cuff was released. The volume of the forearm was allowed to stabilize; then the saline infusion was switched to an infusion of 15 to 30 micrograms per minute of norepinephrine base. The record of the volume of the forearm was observed for three minutes after which the measurement of the pressure-volume curve of the veins was repeated and the volume of the forearm reestablished once again. Arterial occlusion at the wrist was maintained through this sequence.

The effects of environmental and local cooling. Normal young male subjects wearing shorts only were stud-

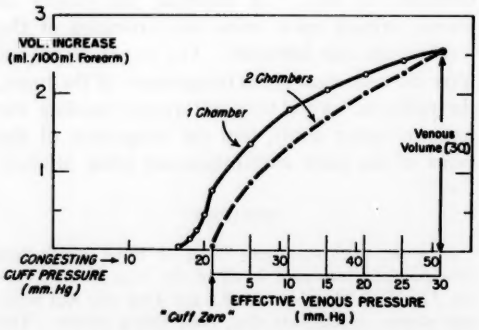

FIG. 2. THE RELATIONSHIP BETWEEN CONGESTING CUFF PRESSURE, EFFECTIVE VENOUS PRESSURE AND VOLUME CHANGE OF THE DISTAL FOREARM SEGMENT *WITH* WATER IN THE PROXIMAL CHAMBER (TWO CHAMBERS) AND *WITHOUT* WATER IN THE PROXIMAL CHAMBER (ONE CHAMBER)

"Cuff zero" and venous volume [30] are also indicated.

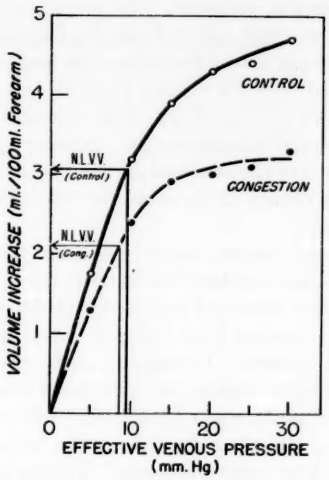

FIG. 3. VENOUS PRESSURE-VOLUME CURVES OBTAINED IN A SINGLE EXPERIMENT DURING CONTROL AND CONGESTION PERIODS

Concomitantly measured venous pressure is used to deduce natural local venous volume (N.L.V.V.) in each case.

ied in the sitting position in a constant temperature room. They were urged to relax but were not allowed to sleep. The warm environment ranged from 29° C. to 32° C. and was continued until digital skin temperature showed no further tendency to rise (usually requiring 30 to 60 minutes). An additional 20 to 30 minutes were utilized to obtain two or three successive venous pressure-volume curves. The cool environment consisted of a room temperature of 18° C. to 21° C., associated with a gentle breeze produced by a fan near the subject. The environment was continued until the venous volume [30] values became stable (60 to 90 minutes). Then two or three more pressure-volume curves were obtained. Eleven of the experiments were started in the warm environment and seven were started in the cool environment. Temperature of the water in the plethysmograph was kept at 32° C. throughout all of these experiments.

The effect of change of local temperature was investigated by maintaining environmental temperature unchanged (29° C.) and reducing the temperature of the water in the plethysmograph. Three venous pressure-volume curves were obtained at a water temperature of 32° C., then three more were obtained after water temperature had been reduced to 18° C.

The effect of congestion of the legs. The same method was used except that the pressure required for the first increment was introduced suddenly so that the initial rate of inscription of the pressure-volume curve of the veins was volume flow of blood. Normal young male subjects were studied on a tilt table in a 30 degree (from horizontal) head-up position. Room temperature was maintained at 25° C. The forearm in the plethysmograph was at the level of the heart. The left antecubital space was at the level of the heart and at the level of the shoulder as well. The membrane of a Statham strain gauge used to measure venous pressure was placed at the level of the right atrium (3). An arterial occluding cuff was inflated on the left wrist during each venous pressure determination. Arterial pressure was determined at five minute intervals by the auscultatory method in the left arm. Mean arterial pressure was considered to be diastolic pressure plus one-third pulse pressure. The rate of the heart was also determined by auscultation at the apex at five minute intervals. Pneumatic congesting cuffs encircled both upper thighs and the left elbow below the needle in the vein. Pneumatic leggings covered the rest of the legs. The cuffs on the upper part of the legs, the cuff on the left forearm and the leggings were inflated from three separate sources. The cuffs on the wrists were inflated from a fourth source. A wide canvas band was used to strap the subject's knees to the table to allow him to relax comfortably. During the initial control period which lasted for 30 to 45 minutes, the leggings and upper leg cuffs were inflated to a pressure which was equivalent to the vertical hydrostatic distance between the knee and the level of the right atrium. Three or more venous pressure-volume curves from the right arm with simultaneous venous pressure from the left arm were recorded during this period. Following this, pressure in the pneumatic leggings was released and simultaneously the three congesting cuffs were inflated to 70 mm. Hg. Then, the pressure-volume curves and venous pressure measurements were repeated at 5 to 10 minute intervals until venous volume [30] became constant. Three more pressure-volume curves were then obtained. Subjects were not allowed to move their legs during the congestion period.

The recovery period was initiated by release of the cuffs on the upper legs and left elbow, followed in 60 seconds by inflation of the leggings and cuffs on the upper legs to the pressure originally used in the control period. Venous pressure-volume curves and simultaneous venous pressures were measured at 5 to 10 minute intervals until the value for venous volume [30] became constant. The last three curves, and their associated venous pressures, mean arterial pressures and pulse rates were averaged to obtain a single value for each function during the control period, the congestion period, and the recovery period.

In separate experiments subjects were studied in the horizontal position utilizing exactly the same procedure as described above.

The venous pressure as measured in the left arm was the natural local venous pressure. This pressure and the concomitantly measured peripheral venous pressure-volume curve were used to deduce the volume of blood present in the venous bed of the limb segment at that pressure. This volume will be referred to hereafter as the *natural local venous volume*. Thus it was possible to calculate the per cent change in natural local venous

volume produced by congestion of both legs and the opposite forearm (Figure 3).

All the experiments were analyzed statistically on the basis of paired data. The variability within the control periods or the experimental periods (congesting or cooling) was investigated by determining the differences between each of the values of venous volume [30] and their mean (means only are tabulated in Tables I, II, and III) for that period. Standard error of the mean, standard error of the difference, and probability were then determined for the group of experiments.

RESULTS

Changes in effective venous pressure and forearm volume resulting from inflation of the proximal cuff by 1 mm. Hg increments while only the distal chamber of the plethysmograph was filled with water were measured on 5 subjects 11 times. A volume change of the distal forearm segment under these circumstances invariably preceded a change in measured effective venous pressure. This discrepancy was such that it was necessary to inflate the cuff by three to five additional 1 mm. Hg increments, before a rise in measured effective venous pressure could be induced. Following this and in the same experiment, water was added to the proximal chamber of the plethysmograph. Volume change of that segment was also recorded. The procedure of inflating the proximal cuff by 1 mm. Hg increments was repeated and it was found that the first change of measured effective venous pressure and the first change of volume of the distal forearm segment then occurred simultaneously. However, a change in volume of the proximal segment invariably preceded a change in its measured effective venous pressure.

Effective venous pressure without pressure in the proximal cuff but with water in the plethysmograph was measured repeatedly in the five subjects. It ranged from 0.5 to 1 mm. Hg and remained constant throughout each experiment. This effective venous pressure value was not influenced by the water level of the plethysmograph provided the pressure of the water exceeded the natural local venous pressure. Volume change in the proximal chamber always preceded effective venous pressure change by three to five 1 mm. Hg increments in cuff pressure. Figure 2 illustrates the results of one of these experiments.

Despite the discrepancies in the time of onset of the response in volume and in pressure in the single chamber plethysmograph, change of venous volume in response to a rise of 30 mm. Hg ef-

TABLE I

Venous volume [30] and blood flow in ml. per 100 ml. forearm tissue in the warm and cool environments, with per cent reduction of each and notation as to which environment the subject was first exposed

Subject	Warm (W) or Cool (C) first	Venous volume [30]		% reduction	Blood flow		% reduction
		Warm	Cool		Warm	Cool	
1	W	1.6	1.4	13	2.9	1.0	66
1	C	2.2	1.4	36	1.4	1.0	29
1	C	3.9	3.2	18			
1	W	4.1	2.7	34			
1	W	3.3	2.5	24			
2	W	2.7	2.2	19	4.6	1.0	78
2	C	2.7	2.0	26	2.5	1.6	36
3	C	3.7	2.5	32	1.2	1.0	17
4	W	3.0	1.8	40	4.6	1.1	76
5	C	3.3	2.7	18			
5	W	2.8	2.0	29			
5	C	2.5	1.1	56			
6	W	3.1	2.6	16			
6	W	4.3	3.7	14			
7	W	2.9	1.4	52			
8	W	4.8	2.8	42			
9	W	3.4	2.2	35			
10	C	0.9	1.3				
Standard error of the means*		0.052	0.071				
Standard error of the difference		0.088					
Mean difference		0.87					
Probability		0.01					

* See text.

TABLE II

Average venous volume [30] and blood flow in ml. per 100 ml. forearm tissue during control (Cl), congestion (Cn), and recovery (Ry) periods with per cent decrease of control value during congestion of venous volume [30], and natural local venous volume (N.L.V.V.) per cent decrease of control value during congestion *

Subject	Venous volume [30] Cl	Cn	Ry	% decrease	Blood flow Cl	Cn	Ry	% decrease N.L.V.V.
1	2.3	2.0		13				
1	2.8	2.3	2.8	18	2.6	1.1	1.2	44
1	2.7	2.3	2.8	15	1.2	1.0	1.1	33
1	2.5	1.7	3.1	32				40
1	2.5	2.0	2.6	20	0.9	0.7	0.9	22
1	3.2	2.2	3.1	31	1.1	0.7	0.9	55
2	4.7	3.3	4.0	30	2.1	1.2	1.6	
2	4.3	3.7	4.3	14	2.0	1.4	1.5	33
2	4.2	3.4		19	2.0	1.5		32
3	1.7	1.4	1.7	18	1.2	0.7	1.1	
3	3.5	2.2	2.9	37				58
4	3.2	2.5	2.8	22	1.9	1.1	1.2	41
4	3.8	3.3	3.9	13	2.8	1.8	1.8	
4	4.7	3.8	4.1	19				
5	2.2	1.8	1.9	18	2.0	1.5	1.7	
5	2.7	2.0	2.7	26	1.5	1.0	0.9	
6	2.4	2.1	2.4	13				
7	2.8	2.5	2.9	11	2.4	1.1	1.3	
8	3.5	2.3	3.3	34	1.5	1.0	1.0	
9	2.5	2.1		16	1.9	1.3		
10	1.7	1.6	1.5	6	3.9	3.4	3.2	
Standard error of the means†	0.029	0.035						
Standard error of the difference	0.045							
Mean difference	0.63							
Probability	0.01							

* Thirty degree head-up position.
† See text.

fective venous pressure was essentially the same with or without water in the proximal chamber (Figure 2).

The intravenous infusion of norepinephrine in 3 subjects on 12 occasions resulted in no change in the volume of the forearm when there was no pressure in the proximal cuff. Venous volume [30] decreased in every instance with infusion of norepinephrine. Average control venous volume [30] was 3.2 ml. per 100 ml. of forearm while venous volume [30] with norepinephrine infusion was 2.4 ml. per 100 ml. of forearm. In two other subjects and one of the above subjects on five occasions, venous volume [30] was also decreased by norepinephrine infusion but the volume of the forearm without pressure in the proximal cuff was also reduced by 0.3 to 0.7 ml. per 100 ml. of forearm. Average control venous volume [30] in these experiments was 1.6 ml. per 100 ml. and average venous volume [30] with infusion of norepinephrine was 1.0 ml. per 100 ml. of forearm.

The effect of environmental and local cooling

Ten subjects were studied 18 times in the warm and cool environments. In the cool environment venous volume [30] was decreased in every instance except one. With the exception of that subject, the average decrease in venous volume [30] was 34 per cent and ranged from 13 to 56 per cent (Table I).

TABLE III

Average venous volume [30] and blood flow in ml. per 100 ml. forearm tissue during control (Cl), congestion (Cn), and recovery (Ry) periods—Horizontal position

Subject	Venous volume [30] Cl	Cn	Ry	Blood flow Cl	Cn	Ry
1	2.2	2.1	2.2	1.0	0.8	0.8
2	3.8	3.5		1.7	1.2	
4	3.1	3.2				
9	2.1	2.4		1.7	2.0	
9	4.0	4.1		2.6	1.9	
9	4.0	4.2	4.1	4.0	2.8	2.9

Six of these experiments on four subjects were carried out with measurements of blood flow (Table I) on the opposite forearm by the venous occlusion plethysmographic method (4). In the three experiments with the room warm first, blood flow fell as room temperature was being lowered (5 to 10 minutes), while venous volume [30] did not decrease for 30 to 60 minutes. In the three experiments done with the room cool first, venous volume [30] rose during exposure to the warm environment, and in two this occurred 5 to 10 minutes before blood flow rose. In the third there was no difference in the onset of the responses.

Two subjects were studied four times in the warm environment with the temperature of the water in the plethysmograph first at 32° C., then at 18° C. Venous volume [30] was less with cool water in all four experiments by an average of 31 per cent, ranging from 18 to 44 per cent.

The effect of congestion of the legs

Ten subjects were studied in 21 experiments in the 30 degree head-up position (Table II). Venoconstriction in the forearm in response to pooling of blood in the veins of the limbs occurred in 20 of the 21 experiments. Blood flow determinations during the congestion period were satisfactory in 16 of these experiments. Constriction of the arterioles, indicated by decreased blood flow in the presence of a slight *rise* in mean arterial pressure, occurred in all 16 experiments. Venous pressure decreased in all of the nine experiments in which it was measured. The decreases at their maximum ranged from 4 to 18 mm. water.

In the 16 experiments in which both constriction of the veins and the arterioles was observed, the onset of constriction of the arterioles occurred within the first 10 minutes of congestion of the limbs and clearly preceded the onset of constriction of the veins in 11. This difference in time of onset ranged from 5 to 35 minutes. The time of onset of these two phenomena could not be distinguished in four experiments, and constriction of the veins clearly preceded constriction of the arterioles in one. The congestion period was 45 to 90 minutes in duration.

During the recovery period venous volume [30] measurements were satisfactory in 17 experiments and blood flow measurements were satisfactory in 14 experiments. Dilatation of the veins relative to the state of constriction observed during the congestion period occurred in all 17 of these experiments. Dilatation of the arterioles occurred in 11 before termination of the experiment, while the constriction of the arterioles induced during the congestion period persisted throughout the recovery period in the remaining three experiments. In 10 of these 11 experiments dilatation of the veins started before dilatation of the arterioles began. In one case there was no apparent difference in the time of onset of dilatation of the veins and the arterioles.

Venous pressure measurements were satisfactory in eight recovery periods of these experiments. In seven of these, sudden reapplication of compression in the leggings resulted in overshoot of venous pressure relative to the subsequent resting level. This overshoot ranged from 7 to 24 mm. of water and lasted from 1 to 3 minutes.

The average pulse rate during the control period was 72 per minute. Pulse rate rose in every experiment during the congestion period to an average maximum level of 80 (the subject with vasovagal syncope is excluded). The average basal level in the recovery period was 70. Average mean arterial pressure was 102, 104, and 104 mm. Hg, respectively, during these three periods.

The percentile reduction in natural local venous volume during the period of congestion was computed in the nine experiments in which venous pressure was measured. It ranged from 22 to 58 per cent and averaged 39.8 per cent. The value exceeded 32 per cent in all cases except one, which was 22 per cent. It was of interest to note that fainting occurred only in this subject. His pulse rate was less than 40 during this episode, suggesting a vasovagal response. The remaining subjects had no serious symptoms during congestion but did complain variously of warmness, sweating, listlessness and annoyance, in that order of frequency.

Six experiments were done in the horizontal position (Table III) without venous pressure measurements. Constriction of the veins occurred in two of these subjects to a mild degree, while no response was evident in the remaining four experiments. Pulse rate, blood flow and arterial pressure were little affected by congestion

of the limbs of subjects in the horizontal position. The congestion period was maintained for 60 to 90 minutes in these experiments.

DISCUSSION

The method utilized for these studies is a further modification (5) of a previously described method (6). The further modification was done in order to approximate more closely *in vivo* the ideal *in vitro* circumstances necessary for measuring the pressure-volume characteristics of a distensible system (7), in this case the veins of the forearm. This ideal situation would have as its starting point a completely empty system at zero or even negative pressure and permit one to raise the pressure in convenient steps while measuring the simultaneously induced volume change with each step. However, it is impractical to use a bloodless venous bed at zero effective pressure *in vivo* because of its attendant physiologic (circulatory arrest) and technical (pressure plethysmography) disadvantages. Therefore, it becomes necessary to demonstrate that changes of volume of the undistended veins induced by a strong venoconstrictor stimulus are small or at least predictable. The points along the venous pressure-volume curve are valid only if it can be demonstrated that associated changes of pressure and volume occur simultaneously.

The phenomenon of volume increase preceding pressure increase when a single chamber plethysmograph was used was probably due to early congestion of the poorly pressurized cone of tissue at the proximal end of the plethysmograph (8). A pressure cone occurs between two segments of forearm subjected to different pressures, with the base at the level of the change in pressure and the apex toward the higher of the two pressures. Thus, there would be no cone within the distal end of the plethysmograph, although there would be a cone beneath the proximal end of the arterial occlusion cuff distal to the plethysmograph.

The total measured increase in volume of the venous system of the forearm (ml. per 100 ml. of tissue per 30 mm. Hg increase in effective venous pressure) was not altered significantly by the presence or absence of the proximal chamber of the plethysmograph. Thus studies of venous volume [30] utilizing a single chamber plethysmograph (5) would not be invalidated by the findings described herein. On the contrary, the larger single chamber plethysmograph has the advantage of smaller variability of results when one subject is to be compared with another (due presumably to the smaller variability from one subject to another of the bone to soft tissue ratio of a longer segment) and is preferable for this type of study. Since the additional refinement of a proximal chamber allows inscription of an accurate pressure-volume curve, local venous volume at low venous pressures may be accurately predicted. The two-chamber plethysmograph, therefore, appears to be superior to the single-chamber plethysmograph for study of acute responses of veins when a subject is used as his own control during a single continuous experiment.

The forearm venous pressure-volume curve is a graphic presentation of the volume of blood that a venous system of 100 ml. of forearm tissue will accept with a given change of effective venous pressure. Constriction of the smooth muscle of the veins results in a *less* distensible system. In addition, venous pressure-volume curves indicate the magnitude of the decrease in local venous volume, at a given effective venous pressure, which has been caused by venoconstriction if the volume of the veins at their lowest effective venous pressure (0.5 to 1.0 mm. Hg) is unaltered by the venoconstriction. When venoconstriction is intense or occurs in an already constricted venous bed, the initial volume of the veins at this low effective venous pressure may decrease. In this case, decrease in venous volume at a given effective venous pressure would be greater than that indicated by the venous pressure-volume curve which has been observed. Moderate venoconstriction of a previously dilated venous bed, induced by infusion of norepinephrine, did not alter the volume of the veins at low effective venous pressure. Leonard and Sarnoff have observed that an unstretched helical section of vein shortens when exposed to a constrictor agent (9). The explanation for the apparently contradictory observations may be that dilated veins at low effective venous pressures may be elliptical in cross-section and may tend to become circular with constriction. This geometric change would allow the cross-sectional area (thus volume of the tube) to remain unchanged despite a decrease in

circumference of the tube. Once the circular configuration was reached, further constriction would result in a decrease in volume even at low effective venous pressure. The observation that venous beds with a low control venous volume [30] tended to diminish in volume at low effective venous pressure after infusion of norepinephrine is consistent with this hypothesis. The length, width and thickness of the unstretched venous system cannot be determined *in vivo* by presently available methods; thus, its length-tension relationships relative to resting length cannot be calculated. This fact does not alter the contribution of the venous pressure-volume curve to a more complete understanding of the physiology of the peripheral veins.

The effect of environmental and local cooling

The responses of the veins to cooling of the environment and to cooling of the extremity locally indicate that it is necessary to control these temperatures during experiments designed for study of the veins. The difference in rate of response (though the direction was the same) of the arterioles and veins is further evidence that the method measures the characteristics of the veins, independently of arteriolar changes.

Greenfield and Patterson (10) found that high environmental temperatures produced no further venodilatation when the experiment was started in a comfortable environment. Although their method was more analogous to the pressure plethysmographic approach (11) rather than to the method presently described, the resulting data can probably be compared at least qualitatively with those obtained by methods reported here. Results obtained with these methods with body warming are essentially the same (12). Further dilatation of veins does not occur despite the marked arteriolar dilatation that occurs with body heating. (This is another example of a difference in the response of the arterioles and veins.) Unlike the arterioles, the peripheral veins appear to be maximally dilated when the subject is comfortably warm.

The effect of congestion of the legs

There is little doubt that the peripheral venous system participates actively in the complex vascular responses to a diminution of effectively circulating blood volume (13–16). Further questions are: 1) What is the time-course relationship of the venoconstriction and the arteriolar constriction? 2) What is the degree of venoconstriction in terms of the venous pressure-volume curve and the resultant change in natural local venous volume?

The intensity of the stimulus employed in these experiments depends upon the volume of blood removed from the parts of the vascular bed not subjected to congestion and the position of the subject. It may be categorized further by the severity of symptoms and signs induced by the congestion of the limbs.

Ebert and Stead (17) measured the volume of blood pooled in both legs and one arm of supine normal subjects by cuffs on these extremities inflated to diastolic pressure. The volume changes obtained were probably similar to those produced in the experiments reported here despite the difference in position of the subjects, since pneumatic leggings were used to prevent dependent pooling of blood during the control periods in the experiments here reported. They found that the blood volume normally present in the head, trunk and one arm was reduced by an average of 720 ml. or 15 per cent, with a range of 13 to 18 per cent.

The signs and symptoms of the subjects and the observations of Ebert and Stead (17) indicate that the intensity of the stimulus to the cardiovascular system was of the order of that produced by an acute reduction of effectively circulating blood volume of 500 to 1,000 ml. when the subject is in the head-up position.

It has been suggested (18) that under most circumstances venous and arteriolar responses occur simultaneously. This implies that the response of veins and arterioles is one of a parallel increase in tone, the degree and rapidity of which depends upon the stimulus. However, under the circumstances of the experiments described here, rigid parallelism of these responses in the forearm was usually absent. With the onset of limb congestion arteriolar constriction began almost at once while venoconstriction not only began later, but required more time to reach maximum intensity. Relief from congestion resulted in just the opposite response inasmuch as arteriolar constriction persisted after remission of venoconstriction.

These differences in rate of response suggest that initial moderate reduction of effectively circulating blood volume of an uptilted subject represents only a mild stimulus to the forearm veins. On the contrary, the same stimulus appeared adequate to induce an immediate response of the forearm arterioles and thus might be considered to be a relatively more intense stimulus to the peripheral arterioles.

Limb congestion in the horizontal position failed to induce significant peripheral vascular responses in these experiments and has failed to alter cardiac output in other experiments (19). Limb congestion in the 30 degree upright position resulted in a dissociation of the venous and arteriolar responses. It is reported that sudden tilting to the upright position causes an immediate (within 50 seconds) constrictor response of a single superficial vein (15). These observations suggest that in man the peripheral arterioles respond to increasing reductions of effectively circulating blood volume at a lower level than do the peripheral veins.

The per cent reduction of natural local venous volume of the right arm induced by pooling of blood was much greater (40 per cent) than the presumed reduction of the vascular volume of the head, trunk, and right arm (15 per cent). This finding implies that the veins of uncongested skeletal muscles did not merely reduce their volume by the amount of blood that was removed but actually tended to move the remaining blood volume centrally. That is, the amount of venoconstriction in the periphery exceeded that of more centrally placed veins.

Sudden restoration of blood volume following congestion of the legs caused a sharp rise in venous pressure to a point above the level that it was to assume later in the recovery period. The finding of venous pressure "overshoots" suggested that the venoconstriction induced by congestion of the limbs persisted for a few moments after relief from congestion.

SUMMARY AND CONCLUSIONS

1. A new two-chamber plethysmographic method for accurately recording the peripheral venous pressure-volume curve in man is described.

2. Using this method, peripheral venoconstriction is found to occur in a cool environment, or after locally cooling the part in the plethysmograph.

3. Arterial constriction commonly precedes venoconstriction on going from a warm to a cool environment while venodilatation usually precedes arteriolar dilatation on going from a cool to a warm environment.

4. Congestion of the legs in the 30 degree (from horizontal) head-up position usually resulted in prompt arteriolar constriction and venous pressure reduction *followed* by peripheral venoconstriction (in the forearm).

5. Relief of congestion usually caused in sequence: 1) venous pressure "overshoot," 2) venodilatation, and 3) arteriolar dilatation.

6. Natural local venous volume of the forearm veins was reduced by two and one-half times the presumed percentile reduction in effectively circulating blood volume. This finding suggests that after congestion of the lower limbs the remaining blood volume was moved centrally by venoconstriction in the periphery which was more intense than that in the central veins.

7. The venous pressure overshoot following restoration of effectively circulating blood volume to "normal" suggested that the venoconstriction that had taken place in response to a reduction of blood volume had persisted for a short time after its restoration.

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SCHOOL OF DENTISTRY
University of Detroit

HEXOSAMINES OF SEROMUCOID[1]

By WILLIAM P. DEISS and LEILA B. HOLMES

(From the Medical Service, Veterans Administration Hospital, and the Departments of Medicine and Biochemistry, Duke University School of Medicine, Durham, N. C.)

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A renewal of interest in seromucoid, or the acid mucoprotein of human serum, was initiated by Winzler, Devor, Mehl, and Smyth in 1948 (1). Since that time many studies of the significance of this fraction have been published (2–4). It is now well established that this carbohydrate-rich protein fraction is increased in the serum in a wide variety of disease states, including acute and chronic inflammation and metastatic malignancies. Decreased levels have been found in nephrosis and cirrhosis. These variations of seromucoid in disease have recently been reviewed by Greenspan (5) and by Winzler (6). In general, it has been postulated that the seromucoid elevations are in some way related to destruction and/or proliferation of connective tissue ground substance, and the reduced levels in nephrosis and cirrhosis represent urinary loss and defective formation of seromucoid, respectively.

While the recent literature is replete with studies of the variation of total seromucoid in disease (2–6), there is considerably less information on variations of the carbohydrate components within this fraction which is very probably quite heterogeneous. There appear to be at least three electrophoretically distinct components in this fraction (7), and there is evidence that the carbohydrate to protein ratio of seromucoid may vary in different diseases (2, 8). If the circulating seromucoid is indeed related to the destructive and/or proliferative changes occurring in the connective tissue ground substance, then qualitative differences in this serum fraction in these clinically distinct diseases might indicate some basic difference in the pathologic process at the tissue level. Both connective tissue ground substance and seromucoid are rich in hexosamine. In the case of ground substance a good deal of information has been accumulated about the mucopolysaccharide composition of various tissues (9). The chondroitin sulfates and the non-sulfated chondroitin contain galactosamine, while hyaluronic acid contains only glucosamine. Both glucosamine and galactosamine have been found in seromucoid (10, 11) but little is presently known of the ratio of these two hexosamines in this fraction in normal subjects or in disease states where the total quantity of seromucoid is altered. The purpose of the present study, therefore, was to determine the quantities of glucosamine and galactosamine in seromucoid in normal subjects and in certain disease states.

METHODS

Perchloric acid was added to 10 ml. of serum diluted to 20 ml. with water, to a final concentration of 0.66 N $HClO_4$ and the filtrate obtained as described by Winzler and co-workers (1). This filtrate was dialyzed against running tap water in the cold room (4° C.) for four to six days, and then lyophilized. The powdered seromucoid was hydrolyzed with 2.0 N HCl for 15 hours in a steam bath. After cooling and filtering, the hydrolysate was placed on a small Dowex 50 ion exchange column from which hexosamine was eluted and determined as described by Boas (12) to give the seromucoid hexosamine. An aliquot of this hexosamine eluate containing approximately 150 micrograms of hexosamine was further separated into glucosamine and galactosamine by a modification (13) of the method of Gardell (14). This involves elution of the hexosamines from a 30 by 0.8 cm. column of Dowex 50 (hydrogen form) using the concentration gradient elution principle (0.6 N HCl in the reservoir with a 500 ml. mixing flask of water) and collecting the eluate with an automatic fraction collector. The hexosamine content of each tube of eluate was determined and plotted as a curve. There was adequate separation of the two hexosamines in all cases. The areas under each peak were calculated by planimetry if there was any overlap of the curves and from these values and the total seromucoid hexosamine both the relative and absolute quantities of each hexosamine were calculated.

When serum protein fractions other than seromucoid were studied, *i.e.*, whole serum protein and the mucoid fractions of Dische and Osnos (15), they were hydrolyzed in 2.0 N HCl and subsequently handled in the same fashion as the seromucoid hydrolysate described above.

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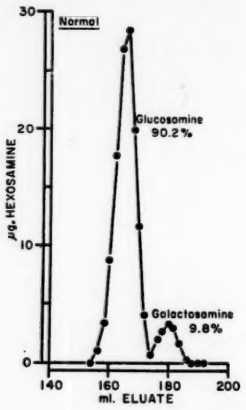

FIG. 1. SEPARATION OF HEXOSAMINES OF NORMAL SEROMUCOID

RESULTS

Figure 1 shows the separation of glucosamine and galactosamine in normal seromucoid. Values for the normal group are shown in Table I. The glucosamine content ranged from 86.3 to 96.8 per cent of the total seromucoid hexosamine, with a mean of 91 per cent. Galactosamine made up the remainder. The total recovery of hexosamine in the two peaks invariably approximated very closely (within ± 4 per cent) the total hexosamine applied to the column. No other peaks were ever noted. The ratio of galactosamine to glucosamine of the normal subjects ranged from 0.03 to 0.16. The total seromucoid hexosamine in these subjects is in the general range of values expected from the findings of Winzler and co-workers (1) that there are about 30 milligrams seromucoid per 100 ml. of serum, 12 per cent of which is hexosamine.

Although the number of patients with connective tissue diseases is limited, these subjects presented a classical example of each disease in its full-blown state of activity. These data are presented in Table I. These subjects in general had some increase in total seromucoid hexosamine but did not demonstrate any significant deviation from the normal galactosamine to glucosamine ratio. In four of these subjects the total serum protein-bound hexosamine was separated into galactosamine and glucosamine and this too did not differ from the normal ratio of 90 to 95 per cent glucosamine and 5 to 10 per cent galactosamine.

The most striking abnormalities of seromucoid galactosamine to glucosamine ratio were found in the three patients with nephrotic stage of glomerulonephritis. Each of these patients was an adult who initially presented the complete clinical picture of this disease state. Pertinent clinical data are in Table II, included in which are the data on a patient with histologically proven amyloid nephrosis secondary to tuberculosis of the spine and lungs (patient No. 1 of the miscellaneous group in Table I). The hexosamine separations in two of these patients, M. I. L. and B. E. L., are shown in Figures 2 and 3, respectively, both before and after some clinical response to therapy. These illustrations demonstrate the altered relationship of galactosamine and glucosamine, when compared with the normal in Figure 1, resulting in the elevated galactosamine to glucosamine ratios indicated in Table I. The average value for this ratio in the three patients with the nephrotic phase of glomerulonephritis, including values before and after some response to therapy, was 0.38. These elevated ratios were apparently due to a decrease in seromucoid glucosamine and an actual increase in galactosamine. The severity of the nephrotic state in these three patients with nephritis did not differ significantly from that in the subject with amyloid nephrosis in terms of edema, proteinuria, hypoproteinemia, and hypercholesterolemia; however, the total seromucoid was distinctly elevated in the latter and the striking disproportion of galactosamine and glucosamine found in the other three patients was not evident.

In the heterogeneous miscellaneous disease group were subjects with elevated and reduced levels of total seromucoid hexosamine. Two patients with chronic glomerulonephritis and parathyroid adenoma, respectively, and the two with myxedema had somewhat increased galactosamine to glucosamine ratios while the remainder had essentially normal ratios.

Figure 4 demonstrates that there is only one hexosamine in a mucoid preparation of whole normal serum. This was a fraction prepared by alkali-alcohol treatment of serum as described by Dische and Osnos (15). When this mucoid fraction was hydrolyzed and fractionated, only one peak was found. When standard galactosamine

TABLE I

Total Serum and Seromucoid Hexosamine and Seromucoid Glucosamine and Galactosamine in Normal Subjects and Patients with Various Diseases

SUBJECTS	Total Serum Hexosamine mg/100 ml.	Seromucoid Hexosamine mg/100 ml.	% of Seromucoid Hexosamine as Glucosamine	% of Seromucoid Hexosamine as Galactosamine	Seromucoid Hexosamine mg/100 ml. Glucosamine	Seromucoid Hexosamine mg/100 ml. Galactosamine	Ratio Galactosamine:Glucosamine
NORMAL GROUP							
1). H.V.N.		2.94	94.2	5.8	2.77	0.17	0.06
2). W.P.D.		3.09	90.2	9.8	2.79	0.30	0.11
3). H.F.	72.2	1.40	86.3	13.7	1.21	0.19	0.16
4). P.J.O.		3.15					
5). S.R.M.		2.40					
6). P.E.P.	83.1	3.49	95.1	4.9	3.32	0.17	0.05
7). C.H.W.	96.6	5.07	87.2	12.8	4.42	0.65	0.15
8). A.C.		2.62	88.1	11.9	2.31	0.31	0.13
9). R.R.M.	70.1	3.6	96.8	3.2	3.48	.12	0.03
Average		3.08	91.13	8.87	2.90	0.27	0.10
CONNECTIVE TISSUE DISEASES							
1). B.U.R. (Scleroderma)		4.00	88.0	12.0	3.52	0.48	0.14
2). L.E.W. (Scleroderma)	76.0	4.00	88.9	11.1	3.55	0.45	0.13
3). C.A.R. (P.A.N.)		7.58	90.3	9.7	6.84	0.74	0.11
4). M.O.O. (D.L.E.)		3.60	89.2	10.8	3.21	0.39	0.12
5). K.I.G. (Rheum. Arth.)	100.2	3.94	84.6	15.4	3.33	0.61	0.18
6). W.O.R. (Rheum. Arth.)		6.76	96.3	3.7	6.51	0.25	0.04
7). S.I.L. (Rheum. Spond.)		7.04	85.7	14.3	6.03	1.01	0.17
Average		5.27	89.0	11.0	4.71	0.56	0.13
NEPHROTIC SYNDROME							
1). M.I.L. Relapse	97.6	2.60	69.7	30.3	1.81	0.79	0.44
Partial (Steroid) Remission		3.08	76.9	23.1	2.37	0.71	0.30
2). B.E.L. Relapse	100.2	1.30	53.1	46.9	0.69	0.61	0.88
Partial Remission	110.1	2.85	73.2	26.8	2.04	0.76	0.37
3). M.C.H. Relapse	41.3	1.30	72.3	27.7	0.94	0.36	0.38
Partial (Steriod) Remission	65.4	1.57	83.6	16.4	1.31	0.26	0.20
Average		2.12	71.5	28.5	1.54	0.58	0.38
MISCELLANEOUS DISEASES							
1). A.B.N. Amyloid Nephrosis		6.07	85.6	14.4	5.19	0.87	0.17
Terminal Uremia	93.0	7.87	85.3	14.7	6.70	1.17	0.17
2). H.A.R. Acute Nephritis		5.34	92.5	7.5	4.94	0.40	0.08
3). S.N.A. Chr. Nephritis		2.28	81.2	18.8	1.85	0.43	0.23
4). J.O.M. Chr. Nephritis with Osteitis Fibrosa Cystica		5.15	92.0	8.0	4.74	0.41	0.09
5). B.R.E. Parathyroid Adenoma		6.64	83.2	16.8	5.52	1.11	0.20
6). S.K.N. Sarcoid with hypercalcemia	94.8	8.37	92.4	7.6	7.73	0.64	0.08
7). F.I.T. Cirrhosis	85.1	4.20	96.2	3.8	4.04	0.16	0.04
8). C.O.N. Cirrhosis	23.7	2.40	97.3	2.7	2.34	0.06	0.03
9). H.A.R. "Stiff Man Syndrome"	65.8	2.10	98.5	1.5	2.07	0.03	0.01
10). R.O.W. Diabetes	70.2	1.70	96.8	3.2	1.65	0.05	0.03
11). H.U.N. Hyperthyroidism	101.6	2.19	93.6	6.4	2.05	0.14	0.07
12). C.H.E. Myxedema	53.4		83.7	16.3			0.19
13). P.E.E. Myxedema	75.0	3.29	81.2	18.8	2.57	0.62	0.24
14). L.E.F. Melanosarcoma	114.7		89.6	10.4			0.11
15). T.H.O. Breast Cancer	113.0		94.7	5.3			0.06

was added to an aliquot of this mucoid hydrolysate, two peaks appear. This was found to be true of both fractions I and II, containing 57 and 8 per cent, respectively, of the total serum hexosamine in both normal and nephrotic individuals. This is taken as evidence that glucosamine is the only hexosamine present in these two fractions.

DISCUSSION

The observations of this study indicate that, though the seromucoid levels are increased in connective tissue diseases and many other disease states in the miscellaneous group, there is little change in the galactosamine: glucosamine ratio. This could mean that there is simply an increase in proliferation and/or destruction of the same ground substance components which are normally being synthesized and degraded.

An explanation is needed for the abnormal ratio of galactosamine and glucosamine in the nephrotic phase of glomerulonephritis. While it is not proven it seems likely that the decreased levels of seromucoid glucosamine which were noted in this study and which account for the reduction of total seromucoid hexosamine are most logically attrib-

TABLE II

Clinical and Laboratory Data on the Nephrotic Patients

Subjects	Clinical Data	Therapy	Cholesterol	TSP	A	G	Urine Protein
M.I.L.	Edematous	0	400	5.0	1.8	3.2	5.3 g/l
	15 lb. wt. loss	40 mg. prednisone daily x 3 weeks	460	5.3	2.3	3.0	3.7 g/l
B.E.L.	Edematous	0	800	4.2	1.1	3.1	7.5 g/l
	25 lb. wt. loss	200 mg. Na diet 4 wks.	480	6.2	2.5	3.7	0
MCH	Edematous	0		3.6	1.0	2.6	15.5 g/l
	13 lb. wt. loss	80 mg. prednisone daily x 3 weeks	538	4.7	1.9	2.8	5 g/l
A.B.N.	Edematous, Azotemia	0	430	3.6	1.0	2.6	15 g/l
	No response to R_x. Terminal uremia. Died 2 wks. later	200 mg. Na diet 5 weeks	180	3.8	1.3	2.5	

utable to urinary loss of the glucosamine-containing components of seromucoid. This would explain the return toward normal levels when proteinuria was reduced by therapy. These observations are in accord with the findings of Kelley, Good, and Glick (16), who noted decreased total seromucoid levels in nephrosis which promptly increased as proteinuria lessened.

The other abnormality in seromucoid hexosamine in this disease, the elevated galactosamine, is assumed to represent an increase in the galactosamine-containing components of seromucoid. It is perhaps not surprising that in this disease there should be urinary loss with a decreased serum level of one (the glucosamine-containing) component of the heterogeneous seromucoid fraction while there is an actual increase in the serum level of another (the galactosamine-containing)

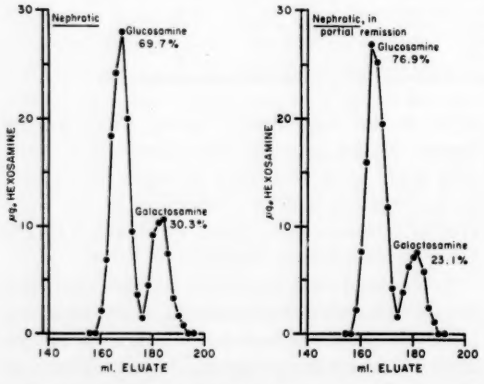

FIG. 2. SEPARATION OF HEXOSAMINES OF SEROMUCOID OF A NEPHROTIC PATIENT (M.I.L.) BEFORE AND AFTER SOME CLINICAL REMISSION

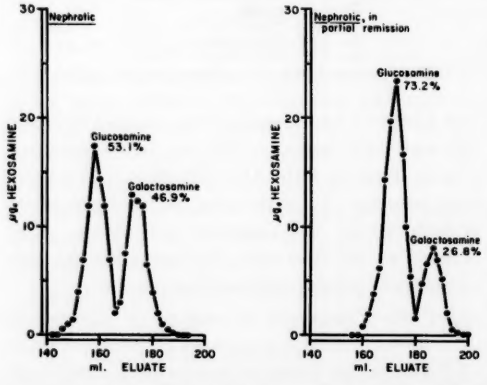

FIG. 3. SEPARATION OF HEXOSAMINES OF SEROMUCOID OF A NEPHROTIC PATIENT (B.E.L.) BEFORE AND AFTER SOME CLINICAL REMISSION

component. There is certainly ample evidence of this same general phenomenon with other serum proteins; notably the striking loss of albumin with hypoalbuminemia along with huge increases in some of the serum lipoprotein fractions. One might infer from this that glucosamine-containing seromucoid is of smaller molecular size then galactosamine-containing seromucoid. The contrast between the low seromucoid levels in the nephrosis of chronic glomerulonephritis and the elevated levels in amyloid nephrosis has been previously noted (5) and remains unexplained. In our patient with amyloidosis the proteinuria was as great as in the other nephrotics. In the former there was an increase in both glucosamine and galactosamine.

Generalizations regarding the values in the miscellaneous group seem inappropriate since there was considerable variation and the numbers of patients with each disease state was quite small. These subjects were included in this study in the process of surveying groups of diseases for abnormalities in seromucoid. One finding which is worthy of further study was the increased galactosamine to glucosamine ratios found in two patients with primary myxedema. This disease has in common with nephrosis the hypercholesterolemia, and perhaps the galactosamine-containing seromucoid is related to the lipid disturbance in these diseases. It is also worth pointing out that in primary myxedema there is, of course, the increase in connective tissue ground substance which may in some way be reflected in the altered galactosamine to glucosamine ratio.

It is of some interest that the galactosamine bound to serum protein does not belong to the mucoid fractions which can be split from the protein readily by mild treatment with alkali. Dische and Osnos, who described this method of liberating mucoid substances from serum protein (15), believe that these two fractions, containing 70 and 15 per cent of the total serum protein bound carbohydrate, represent mucopolysaccharide moieties loosely bound to protein. It is clear from our observations that the hexosamine of both of these two alkali-labile fractions is glucosamine, both in the normal and in the nephrotic. This is at variance with the report that this hexosamine is galactosamine (17) and we have no explanation for this discrepancy, except that the latter authors used a different fractionation scheme for the hexosamines.

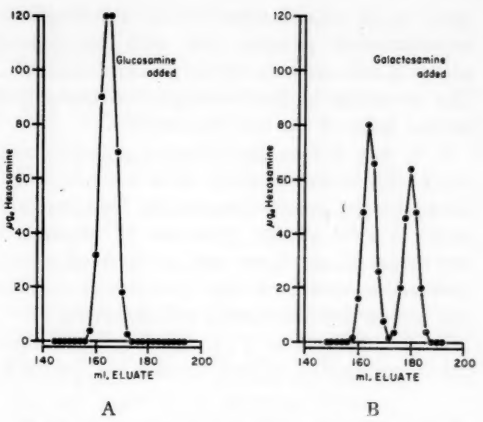

FIG. 4. SEPARATION OF HEXOSAMINE OF NORMAL SERUM MUCOID FRACTION I, WITH GLUCOSAMINE ADDED (A) AND WITH GALACTOSAMINE ADDED (B)

SUMMARY

1. The quantities of glucosamine and galactosamine in seromucoid of seven normal subjects, seven patients with connective tissue diseases, three patients with the nephrotic state of glomerulonephritis and fifteen subjects with miscellaneous diseases were determined, using ion exchange chromatography.

2. The seromucoid of normal subjects contained an average of 91 per cent glucosamine (86 to 97 per cent) and 9 per cent galactosamine (3 to 14 per cent), or a mean galactosamine to glucosamine ratio of 0.10.

3. Although the total seromucoid of the patients with connective tissue diseases was increased, the galactosamine to glucosamine ratio remained essentially the same as in the normal.

4. There was a modest decrease in total seromucoid and of seromucoid glucosamine in the nephrotic phase of glomerulonephritis, whereas there was an increase in the galactosamine resulting in a distinctly increased galactosamine to glucosamine ratio. This abnormality tended to return toward normal during therapy-induced remissions.

5. A slight increase in galactosamine to glucosamine ratio was noted in several of the sub-

jects in the miscellaneous group including two myxedematous patients, one with parathyroid adenoma, and one with chronic glomerulonephritis. The remainder of this heterogeneous group had normal ratios of the two hexosamines.

6. It was shown that, whereas galactosamine was readily demonstrable in whole serum hydrolysates, the two mucopolysaccharide fractions prepared by mild alkaline treatment of serum and containing 57 and 8 per cent of the total serum hexosamine contained only glucosamine. This was true in both the normal and nephrotic.

7. The implications of these findings upon seromucoid metabolism in these diseases are discussed.

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HYALURONATE IN NORMAL HUMAN SYNOVIAL FLUID[1]

By DAVID HAMERMAN and HILDA SCHUSTER

(From the Department of Medicine, Albert Einstein College of Medicine, and the Bronx Municipal Hospital Center, New York, N. Y.)

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Studies on hyaluronate in normal human synovial fluid have been hindered by the high viscosity and small volume of fluid available. Reports on hyaluronate levels in normal synovial fluid, in which a number of different methods have been used, have shown discordant results (1–4).

Several methods were used in the present study to determine the hyaluronate content of normal synovial fluid. Weighing rather than pipetting the small samples of viscous fluid made it possible to measure hyaluronate in 0.25 Gm. of synovial fluid. Hyaluronate was precipitated in mucin clots formed by adding acid or a polyvalent cation to synovial fluid, and was measured indirectly by determining hexosamine in these clots. Since a part of the hexosamine of synovial fluid is not a component of hyaluronate, an important aspect of this work was the demonstration that no non-hyaluronate hexosamine was precipitated in mucin clots, and that no hyaluronate hexosamine remained in the supernatant after a mucin clot was formed. Hyaluronate content of synovial fluid was also measured by determining the fall in hexosamine after hyaluronidase digestion and dialysis of whole synovial fluid. Figures for hyaluronate in synovial fluid as determined by mucin clot technique, and by hyaluronidase digestion and dialysis of synovial fluid agreed, lending confidence in the validity of these methods.

MATERIALS AND METHODS

Deceased subjects were selected who died suddenly after traumatic injury or a vascular accident and who showed no obvious joint disease or systemic illness that might contribute to pathological joint effusions. Synovial fluid was obtained with sterile precautions from both knee joints within six hours after death. Fluids were frozen at −20° C. unless studied within 24 hours, in which case they were stored at 5° C.

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I. Hexosamine determinations on synovial fluid and mucin clots

Synovial fluid. Samples of the viscous synovial fluid were weighed rather than pipetted. Aliquots of synovial fluid (0.10 to 0.15 Gm.) were transferred in glass droppers with tapered ends to pyrex test tubes (13 by 100 mm.). The synovial fluid was diluted to 1 ml. with distilled water and 1 ml. of 8 N HCl was added. The tubes were sealed with a gas-oxygen flame.

Mucin clots. Hyaluronate and protein were precipitated from synovial fluid as mucin clots. Three methods of making mucin clots were studied.

a) Glacial acetic acid. The method of Ropes, Robertson, Rossmeisl, Peabody, and Bauer (5) for bovine synovial fluid was modified. Synovial fluid (0.2 to 0.4 Gm.) was weighed in pyrex test tubes (13 by 100 mm.) and diluted 1:5 with tap water. The diluted synovial fluid was mixed and the tubes placed in a cold room (5° C.) for 15 minutes. A mucin clot was formed in the cold upon addition to the tubes of a volume of glacial acetic acid equal to 1 per cent of the total volume of diluted synovial fluid. The clot adhered to a glass stirring rod used to mix the fluid. After 20 minutes the clots were detached from the stirring rod and the clot and clear supernatant in the test tube centrifuged at room temperature. The supernatant fluid was decanted and the precipitated mucin was washed once with cold distilled water, once with absolute alcohol and dried over P_2O_5 in a vacuum. Two ml. of 4 N HCl was added and the tubes were sealed.

b) Ten per cent (v/v) acetic acid (6). The above procedure was followed. A volume of 10 per cent acetic acid equal to 2.5 per cent of the total volume of 1:5 diluted synovial fluid was used to form a mucin clot.

c) Hexaethylenediamine-hexoltetracobalt (III) nitrate, hereafter referred to as brown salt, is a brown cobalt salt recrystallized three times (7). The cation has a charge of +6 and its formula is

$$Co\left(\begin{matrix}HO\\HO\end{matrix}>Co\ en_2\right)_3\ (NO_3)_6.$$

Synovial fluid (0.2 to 0.4 Gm.) was weighed in pyrex tubes (15 by 125 mm.) and diluted 1:20 with tap water. A volume of a 1 per cent (w/v) solution of brown salt was added equal to 6 per cent of the total volume of diluted synovial fluid.

The sealed tubes were hydrolyzed at 100° C. for six and one-half hours. The tubes were opened, the contents were filtered through Whatman No. 1 paper and were diluted to 25 ml. with distilled water. Aliquots were taken for hexosamine and nitrogen determinations.

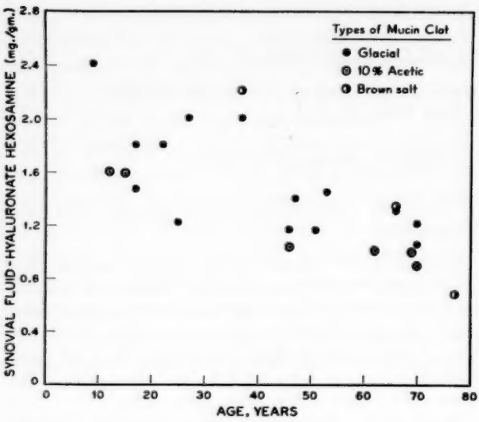

FIG. 1. LEVELS OF HYALURONATE HEXOSAMINE IN SYNOVIAL FLUID OF NORMAL SUBJECTS AT VARIOUS AGES

Hexosamine was determined by the Elson and Morgan procedure as modified by Bilow and Hatch (8). The Kjeldahl procedure (9) was used for nitrogen analysis.

The error in duplicate mucin precipitations and hexosamine analyses was less than 5 per cent.

II. Hyaluronidase digestion of synovial fluid and mucins

Preparation—Synovial fluid. The hyaluronate hexosamine content of synovial fluid was determined by forming a mucin clot and measuring the hexosamine in the clot. Aliquots (0.5 to 1.0 Gm.) of synovial fluid were then diluted by addition of buffer (0.1 M NaCl and 0.1 M $NaHCO_3$, pH 8.1) to reduce the hyaluronate hexosamine content of the synovial fluid to about 0.28 mg. per Gm. The diluted synovial fluid was dialyzed in buffer at 5° C. for three days with daily changes of buffer. Hexosamine was determined in 0.25 Gm. aliquots of this diluted dialyzed synovial fluid and the hyaluronate hexosamine of this fluid was calculated as follows (hexosamine is abbreviated as hx.):

Hx. of diluted dialyzed synovial fluid × per cent hyaluronate hx. in whole synovial fluid equals hyaluronate hx. of diluted dialyzed synovial fluid.

Preparation—Mucins. Aliquots of synovial fluid were weighed in flat bottom centrifuge tubes (40 ml. capacity) and mucin clots were formed by the techniques described. The mucin clots were washed twice with cold distilled water. The hexosamine concentration was adjusted to 0.28 mg. per Gm. by adding buffer, and the mucins were dissolved in the buffer by constant stirring for 24 hours at 5° C. The hexosamine content of dissolved mucin was equivalent to hyaluronate hexosamine. After determination of their viscosity, and prior to hyaluronidase digestion, the mucin solutions were dialyzed against buffer for two days at 5° C.

Preparation—Supernatants remaining after mucin precipitation. The mucin supernatants were first dialyzed against buffer for two days at 5° C. NaCl was added to the supernatant of the brown salt mucin so that excess cobalt would be displaced during dialysis. Following dialysis in buffer, all supernatants were dialyzed at 5° C. for two days in distilled water. The contents of the dialysis casing were emptied into beakers and evaporated to dryness over P_2O_5 *in vacuo*. Buffer was added to the beakers and the supernatant material dissolved by mixing. The hexosamine concentration of this material was about 0.2 mg. per Gm. or less.

Hyaluronidase digestion. Three ml. of diluted dialyzed synovial fluid or dissolved dialyzed mucins was brought to pH 4.7 to 4.8 with 10 per cent acetic acid. Glacial acetic acid was used to lower the pH of the mucin supernatants since it was necessary to avoid further dilution of the small quantity of hexosamine in the supernatants. The acidified fluids were then divided into three parts by distributing 1 ml. aliquots to a stoppered glass vial (8 ml. capacity). The contents of one vial served as a control and 0.1 ml. of the NaCl–$NaHCO_3$ buffer was added. To the contents of a second vial was added 0.1 ml. of the same buffer containing 75 turbidity reducing units (TRU) of testicular hyaluronidase (Wyeth). One-tenth ml. of this buffer containing 75 TRU of streptococcal hyaluronidase was added to the fluid in a third vial. The vials were shaken and then incubated at 37° C. for 72 hours. The contents of the vials were then thoroughly mixed and dialyzed on a shaker platform in three separate beakers at 5° C. for three days with changes of buffer twice daily. Aliquots up to 1.0 Gm. of the dialyzed material were weighed in pyrex tubes (13 by 100 mm.) and hexosamine determinations were carried out. Hexosamine of the dialyzed control less hexosamine of the hyaluronidase treated dialyzed material was a measure of hyaluronate hexosamine.

Hyaluronidase activity was also tested against well defined substrates to define accurately such variables as the extent of enzymatic digestion and the hexosamine blank contributed by the enzymes. These substrates included: 1) 4 per cent (w/v) gamma globulin dialyzed against buffer; 2) 1 ml. (50 mg.) of heparin (Vitarine Co.) diluted to 50 ml. with buffer; 3) hyaluronate prepared (5) from bovine synovial fluid, partially purified (hexosamine, 23 per cent; nitrogen, 6 per cent) and dissolved in buffer to a concentration of about 0.3 mg. hexosamine per Gm.; and 4) potassium chondroitin sulfate (24 per cent hexosamine) prepared from the crystalline calcium salt and similarly dissolved.

III. Viscosity measurements

These studies were carried out on diluted dialyzed synovial fluids and on dissolved mucins at comparable hyaluronate hexosamine concentrations (0.28 mg. per Gm.). Viscosity of these materials was measured at 37° C. using Ostwald viscosimeters of 2 ml. capacity and a flow time for buffer of 24 seconds.

IV. Bovine synovial fluid

Studies of this material form a minor part of this report. Fluid obtained from the astragalo-tibial joints of freshly slaughtered animals was clarified by filtration through glass wool and stored at − 20° C. The concentration of hyaluronate hexosamine in this fluid was about 0.18 mg. per Gm. Before studies of the extent of hyaluronidase digestion were carried out, bovine fluid was concentrated *in vacuo* until the hyaluronate hexosamine concentration was raised to about 0.28 mg. per Gm., and was then dialyzed in buffer.

RESULTS

I. Analytical data

Table I presents the total hexosamine of synovial fluids, and hexosamine in mucin clots precipitated from synovial fluids of the 23 normal subjects. Total nitrogen, mucin nitrogen and nitrogen to hexosamine ratios were measured in some subjects. In all cases but two, mucin hexosamine, calculated as mg. per Gm. of synovial fluid, was lower than the total hexosamine of synovial fluid. As shown in data presented below, mucin clots formed from synovial fluid contained all the hyaluronate hexosamine and only non-hyaluronate hexosamine remained in the supernatant. A figure for hyaluronate in synovial fluid may be obtained by dividing mucin hexosamine by 40 per cent, the hexosamine content of protein free hyaluronate. Where the three methods used to form mucin clots can be compared, figures for hexosamine in the clots are similar. Ten per cent acetic acid at times led to incomplete precipitation of the hyaluronate as judged by lower mucin hexosamine values. There were slight differences in the analysis of the clots with respect to the nitrogen to hexosamine ratios.

Levels of hyaluronate hexosamine in normal synovial fluid of subjects ranging in age from 9 to 77 years are shown in Figure 1. Values prior to age 40 were somewhat higher than the hyaluronate hexosamine levels after this age.[2]

II. Hyaluronidase digestion

A necessary condition for the digestion of susceptible substrates to *dialyzable* units by testicular hyaluronidase was that the pH of these substrates be below 5 (70 year old subject, Table II). At higher pH levels, testicular hyaluronidase markedly reduced the *viscosity* of the substrates but did not digest the substrates to dialyzable units. Material to be digested by hyaluronidases was adjusted to a pH of about 4.7. Streptococcal hyaluronidase depolymerized susceptible substrates to dialyzable units over a broader pH range, but was somewhat more effective in this respect at pH 4.7 than at 6.8.

TABLE I

Hexosamine and nitrogen in normal synovial fluid at various ages *

Age	Total hx.	Hyaluronate hx.		Total N.	Mucin N.	N./hx. ratio
	mg./Gm.	*clot*	*mg./Gm.*	*mg./Gm.*	*mg./Gm.*	
9	2.59	Gl.	2.42			
12	1.61	10% A.	1.61	3.34	2.05	16.4
15	1.60	10% A.	1.60		2.18	17.5
		Br.	1.55			
17	1.71	Gl.	1.48			
		Br.	1.45			
17	2.05	Gl.	1.82			
		Br.	1.75			
22		Gl.	1.82		2.28	16.1
	2.26	10% A.	1.81	4.35		
		Br.	1.84			
25		Gl.	1.23		1.49	15.5
	1.46	10% A.	1.27	3.23	1.85	18.7
		Br.	1.20		1.44	13.4
27		Gl.	2.02		2.29	14.5
	2.03	10% A.	1.89	4.41	2.66	18.0
		Br.	1.86		2.73	16.8
37	2.53	10% A.	2.11			
		Br.	2.23			
37	2.28	Gl.	2.02			
46	1.39	10% A.	1.04	3.93	1.92	23.7
46	1.39	Gl.	1.17			
		Br.	1.18			
47		Gl.	1.41		1.71	15.5
	1.68	10% A.	1.48	3.14	2.21	19.2
		Br.	1.35		1.50	12.2
51		Gl.	1.17			
	1.47	10% A.	1.01			
		Br.	1.22			
53		Gl.	1.47			
	1.88	10% A.	1.49			
		Br.	1.48			
62	1.39	10% A.	1.02		1.72	21.5
66	1.58	10% A.	1.15			
		Br.	1.35			
66	1.80	Gl.	1.32	4.46	1.93	18.6
69	1.20	10% A.	0.99			
		Br.	1.00			
70	1.12	10% A.	0.89			
70		Gl.	1.22		1.54	16.1
	1.60	10% A.	1.26	3.46	2.02	20.4
		Br.	1.23			
70	1.37	Gl.	1.05			
77	1.01	10% A.	0.69	2.94	1.24	23
		Br.	0.68		1.29	22

* Abbreviations are as follows: Hx., hexosamine; N., nitrogen; Gl., glacial acetic acid; 10% A., 10% acetic acid; Br., brown salt.

[2] Since submitting this paper for publication, additional determinations of hyaluronate hexosamine levels in synovial fluid have been made: Age 33, 1.98 mg. per Gm.; age 52, 1.53 mg. per Gm.; age 85, 0.79 mg. per Gm.

TABLE II

Results of hyaluronidase digestion of synovial fluids and mucins *

Age	Sample	[1]†	pH	Control hx.	Enzymatic digestion and dialysis: After 75 TRU T. Hyd. hx.	Hx. rendered dialyzable	After 75 TRU S. Hyd. hx.	Hx. rendered dialyzable
				mg./Gm.	*mg./Gm.*	%	*mg./Gm.*	%
	Chondroitin sulfate		4.8	0.220	0.008	97	0.227	0
	Bovine hyaluronate		4.8	0.240	0.018	93	0.032	87
	Heparin		5.2	0.141	0.151	0	0.155	0
	γ Globulin		4.8	0.419	0.410	0	0.412	0
17	Synovial fluid	86	6.8	0.296			0.261	12
	Br. mucin		6.8	0.272			0.045	83
22	Synovial fluid	81	4.8	0.279	0.046	84	0.065	77
	Gl. mucin		4.7	0.230	0.017	93	0.030	87
	10% A. mucin		4.8	0.243	0.024	90	0.035	86
	Br. mucin		4.7	0.253			0.033	87
25	Synovial fluid	85	4.6	0.250	0.040	84	0.079	69
47	Synovial fluid	84	4.8	0.343	0.044	87	0.161	54
66	Synovial fluid	73	4.8	0.334	0.111	67	0.278	17
70	Synovial fluid	76	6.5	0.272	0.217	20	0.077	72
	Synovial fluid	76	4.7	0.255	0.067	74	0.077	70
	Gl. mucin		6.8	0.258			0.049	81
	Gl. mucin		4.7	0.240	0.027	89	0.030	88
	10% A. mucin		4.7	0.228	0.024	90	0.028	88
	Br. mucin		4.7	0.253	0.021	92	0.031	88
	Gl. sup't		4.7	0.124	0.120	3		
	10% A. sup't		4.5	0.177	0.165	7		
	Br. sup't		4.6	0.122	0.117	4		

* Abbreviations are as follows: T. Hyd., testicular hyaluronidase; S. Hyd., streptococcal hyaluronidase; sup't, supernatant; TRU, turbidity reducing units.

† [1] equals per cent of total hexosamine of synovial fluid that is hyaluronate hexosamine determined by hexosamine in glacial acetic acid mucin.

Standard substances. A number of experiments were carried out to test the specificity of the hyaluronidases and the extent to which they hydrolyzed known substrates. Table II shows these results. Protein free chondroitin sulfate was digested to the extent of 97 per cent with testicular hyaluronidase but was not affected by the streptococcal enzyme. Hyaluronate prepared from bovine synovial fluid, partially purified (nitrogen to hexosamine ratio of 3) but still containing some protein, showed a fall in hexosamine of 93 per cent after testicular hyaluronidase digestion and 87 per cent after the use of the streptococcal enzyme. This apparent difference in the degree of digestion of hyaluronate by the two enzymes may represent a somewhat greater artificial "blank" in hexosamine analysis contributed by the streptococcal enzyme itself, and probably does not represent less complete digestion of the substrate by streptococcal hyaluronidase. The mucopolysaccharide heparin was not digested by either of the hyaluronidases. Absence of proteolytic activity of the hyaluronidase preparations was shown by their lack of effect in reducing hexosamine content of gamma globulin.

Synovial fluid. Incubation of synovial fluid with hyaluronidase and subsequent dialysis removed that part of the total hexosamine that was hyaluronate hexosamine. The extent of digestion agreed with the estimate of hyaluronate hexosamine in synovial fluid as determined by hexosamine in mucin clots. Table II gives this data and shows that there is very close agreement between the hyaluronate hexosamine of synovial fluid as estimated by glacial acetic acid mucin clots, and that estimated by the extent of digestion of synovial fluid with testicular hyaluronidase at pH 4.7. Streptococcal hyaluronidase in some cases gave very similar results, but at times, as shown in the cases of the 17 and 66 year old subjects, incompletely digested hyaluronate in synovial fluid. The reason for this is not certain but may be due to the presence in these fluids of antibodies to streptococcal hyaluronidase. The very remote

possibility that hyaluronate was not the anionic mucopolysaccharide in these fluids was excluded by noting that streptococcal hyaluronidase completely digested mucins precipitated from these synovial fluids (17 year old subject, Table II).

Mucins. Mucin clots contained all the hyaluronate hexosamine of synovial fluid. Hexosamine in mucins was digested to dialyzable units by streptococcal hyaluronidase. The extent of digestion of the mucins by streptococcal and by testicular hyaluronidase corresponded exactly to that observed in the digestion of purified bovine hyaluronate (Table II).

Further evidence that all the hyaluronate was precipitated from synovial fluid when mucin clots were formed was shown by failure of hyaluronidase digestion and dialysis to reduce the hexosamine in the remaining supernatants (70 year old subject, Table II). The possibility was tested that hyaluronidase activity was inhibited in the supernatant fluid. Glacial acetic acid mucin added to a supernatant was digested by hyaluronidase to the extent of 90 per cent.

III. Viscosity studies

Relative viscosities of synovial fluids diluted with buffer and dialyzed against buffer and mucins dissolved in buffer are shown in Table III. These studies were carried out on fluids of the same ionic strength, pH, and hyaluronate hexosamine concentration. The data show no apparent change in viscosity with aging, but the limitations of this type of measurement in detecting minimal but critical changes in hyaluronate are recognized (10).

Brown salt mucin had a higher relative viscosity than mucins prepared by acidification of synovial fluid. At equivalent hyaluronate hexosamine concentrations the viscosity of the brown salt mucin was equal to the viscosity of the original synovial fluid. Binding of cobalt ions by the brown mucin did not contribute to the higher viscosity noted; after addition of NaCl and dialysis against buffer to remove cobalt ions, the viscosity of the brown mucin was unchanged.

TABLE III

Relative viscosities of normal synovial fluids dialyzed in buffer, and mucins dissolved in buffer, at equivalent hyaluronate hexosamine concentration

	Dialyzed synovial fluid			Dissolved mucins		
Age	Total hx.	Hyaluronate hx.*	η		Hx.	η
	mg./Gm.	*mg./Gm.*			*mg./Gm.*	
17	0.31	0.29	7.3	Br.	0.26	5.4
17	0.32	0.28	7.3			
				Gl.	0.27	3.4
				10% A.	0.29	3.7
22	0.36	0.29	7.4	Br.	0.30	7.0
				Gl.	0.28	3.8
27	0.28	0.28	5.8	10% A.	0.28	4.8
				Br.	0.28	5.7
37				Gl.	0.29	5.1
46	0.33	0.28	6.4			
66	0.41	0.28	5.5			
				Gl.	0.29	5.4
70	0.34	0.26	6.8	10% A.	0.27	5.0
				Br.	0.29	6.8

* Calculated from the per cent of the total hexosamine of synovial fluid found in glacial acetic acid mucin clots.

IV. Bovine synovial fluid

The results of a number of experiments on bovine synovial fluid are summarized in Table IV. On the basis of the fall in hexosamine following hyaluronidase digestion and dialysis of synovial fluid, hyaluronate hexosamine accounted for a little less than one-half the total hexosamine of synovial fluid. Non-anionic mucopolysaccharides containing hexosamine were precipitated to some extent in mucins, particularly those formed with 10 per cent acetic acid and brown salt. The nitrogen to hexosamine ratio of brown salt mucin

TABLE IV

Studies on bovine synovial fluid

	Hx.	% of total synovial fluid hx. in mucin	Hx. rendered dialyzable after hyaluronidase		Nitrogen	N./hx. ratio
			S. Hyd.	T. Hyd.		
	mg./Gm.		%	%	*mg./Gm.*	
Synovial fluid	0.37		47	45	2.18	
Gl. mucin	0.18	49	77	79	0.23	16.3
10% A. mucin	0.20	54			0.37	23.7
Br. mucin	0.20	54			0.65	39.5

TABLE V

Levels of hyaluronate in normal human synovial fluid

Reference	Hyaluronate	Hyaluronate hx.	Method
	mg./ml.	*mg./ml.*	
(1)	0.13		Turbidity
(2)	(2.17)*	0.87	Hexosamine determination in glacial acetic acid mucin clots
(3)	2.97	(1.19)	Hyaluronidase digestion of synovial fluid. Precipitation of non-hyaluronate hexosamine with trichloracetic acid and hexosamine determination on supernatant
(4)	1.71	(0.68)	Determination of hexuronic acid in synovial fluid after removal of some protein with chloroform
Present authors	(3.60)	1.44	Measurement of hexosamine in mucin clots
			Measurement of hexosamine in control and hyaluronidase digested and dialyzed synovial fluids

* Figures in parentheses are calculated from authors' data. The figure of 40 per cent hexosamine in hyaluronate is assumed.

shown in Table IV was higher than values of about 28 observed in earlier experiments on mucins similarly formed in fresh fluid. The higher ratio shown may represent increased precipitation of protein containing non-hyaluronate hexosamine when brown salt was used to form a mucin in fluids stored for some time.

DISCUSSION

An attempt has been made to provide accurate methods for determining hyaluronate levels in normal human synovial fluid. The first point which required attention was the method by which samples of synovial fluid were taken for analysis. Small volumes of highly viscous fluid could not be delivered accurately by pipetting. Measurements were therefore made on the basis of the weight rather than the volume of the synovial fluid. The density of diluted synovial fluid was found not to differ from water within the limits of the precision of the method. Grams and milliliters may therefore be used interchangeably.

All the hyaluronate hexosamine of synovial fluid was precipitated in a mucin clot formed by adding acetic acid or a polyvalent cation to synovial fluid. Non-hyaluronate hexosamine remained in the supernatant and accounted for about 15 per cent or less of the total hexosamine of joint fluid, as previously noted by Sundblad (3). This non-hyaluronate hexosamine was presumably a component of non-anionic mucopolysaccharides. For quantitative precipitation of hyaluronate, glacial acetic acid was preferred rather than 10 per cent acetic acid. A complex cobalt salt, termed brown salt in this paper, with a charge of + 6, also precipitated hyaluronate from synovial fluid. This salt was used by Shatton and Schubert in their work with bovine synovial fluid (11). Mucins precipitated by the brown salt and by glacial acetic acid were similar with respect to hexosamine content and nitrogen to hexosamine ratio. All the hexosamine in mucin clots was digested and rendered dialyzable by streptococcal hyaluronidase. No hexosamine was lost after hyaluronidase digestion and dialysis of mucin supernatants.

Determining the fall in hexosamine following hyaluronidase digestion and dialysis of whole synovial fluid was an additional method to measure accurately hyaluronate levels in synovial fluid. These results agreed closely with those obtained by the mucin clot method.

The average hyaluronate content of normal human synovial fluid in the cases studied was 3.6 mg. per Gm. This figure may be compared with data of other workers in Table V. The average hyaluronate level presented in this paper is higher than any of the figures of other workers. One reason for this may be due to weighing rather than pipetting samples of synovial fluid. Sundblad (3) estimated that the normal range of hya-

luronate in human synovial fluid was 2 to 4 mg. per ml. By contrast with normal human fluid, hyaluronate in bovine synovial fluid was 0.45 mg. per Gm. and hyaluronate hexosamine constituted about 45 per cent of the total hexosamine, as has been previously noted (12–14), instead of 85 per cent as found in human joint fluid. The physical properties of these fluids cannot be compared unless the hyaluronate levels are made equivalent.

Weighing samples of synovial fluid has made it possible to examine the fluid from individual knee joints of subjects of various ages. Generally higher values of hyaluronate have been noted in synovial fluid of subjects before the age of 40. These findings are of interest because articular cartilage of synovial joints has been shown chemically and histologically to contain less chondroitin sulfate with aging (15) or with degenerative arthritis (16–17). Workers whose methods are outlined in Table V have measured hyaluronate concentration in synovial fluid of patients with degenerative arthritis. Compared with normal figures, lower levels of hyaluronate [1.4 mg. per ml. (3)], equivalent levels [2.0 mg. per ml. (2)] and higher values [2.1 mg. per ml. (4)] have been found. Two patients with severe degenerative arthritis of the knees were studied by the present authors. Hexosamine was determined in mucin clots formed by adding glacial acetic acid to the synovial fluid of these two subjects. The accuracy of this technique for measuring hyaluronate in these pathological synovial fluids was established by methods similar to those used for normal fluids described in this paper. Hyaluronate levels of 2.0 mg. per ml. and 1.8 mg. per ml. were found in the joint fluid of these subjects aged 54 and 59, respectively. Values for normal subjects in this age range were about 3 mg. per ml. Articular cartilage and synovial fluid may thus show an analogous decrease in their anionic mucopolysaccharide concentration in the course of degenerative joint changes.

SUMMARY

1. Methods have been described to measure hyaluronate in very small quantities of human synovial fluid. Hyaluronate was precipitated in mucin clots formed by adding acid or a polyvalent cationic cobalt salt to weighed aliquots of synovial fluid and was measured indirectly by determining hexosamine in these clots.

2. These clots contained all the hyaluronate hexosamine of synovial fluid and all the hexosamine in these clots was digested and rendered dialyzable by streptococcal hyaluronidase. Non-hyaluronate hexosamine remained in the supernatant and was not digested by hyaluronidase.

3. Hyaluronate in whole synovial fluid was also determined by measuring the fall in hexosamine after hyaluronidase digestion and dialysis. These figures for hyaluronate agreed with those obtained by the mucin clot technique.

4. The average hyaluronate concentration of normal human synovial fluid was 3.6 mg. per Gm. Hyaluronate hexosamine constituted 85 per cent or more of the total hexosamine of synovial fluid. By contrast, hyaluronate content of bovine synovial fluid was 0.45 mg. per Gm. and hyaluronate hexosamine constituted about 45 per cent of the total synovial fluid hexosamine.

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THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON WATER AND ELECTROLYTE DISTRIBUTION [1]

By A. Y. SWEET, M. F. LEVITT, AND H. L. HODES WITH THE TECHNICAL ASSISTANCE OF H. HABER AND B. KURZMAN

(From the Department of Pediatrics and Medicine, the Mount Sinai Hospital, New York, N. Y.)

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Previous evidence has indicated that in dog and man, the administration of adrenal hormones effects a temporary shift of sodium, chloride, and water in iso-osmotic proportions into the extracellular fluid (1, 2). This conclusion was based on the assumption that inulin afforded a reliable measure of the volume of the fluid phase of the extracellular compartment (3). It has been contended that after the administration of adrenal hormones a molecule like inulin might enter areas from which it was previously excluded (4). In this event, the increased volume of distribution of inulin would not reflect an expanded extracellular volume but rather a change in tissue permeability to inulin. To test the validity of the original conclusion, similar experiments were repeated in monkeys. In this protocol inulin spaces were measured before and after five days of desoxycorticosterone acetate (DCA) administration to monkeys. As in the experiments previously cited (1, 2), the animals were maintained on a low salt intake so that any expansion of the inulin space could not be attributed to retention of salt. In these experiments, however, sodium, chloride, potassium, and water content of muscle were studied in control animals and in those subjected to DCA administration. Tissue electrolyte composition was measured to determine whether the expanded inulin space would be associated with a comparable increase in sodium and chloride content of muscles. Such a correspondence would strongly favor the hypothesis that the extracellular volume had actually increased. Alternatively, it would be difficult to accept the presence of an expanded extracellular volume without a proportional increase in the salt content of muscle.

It has been demonstrated that the stimulus of severe and uncompensated acidosis evokes a rapid transfer of sodium from bone and tendon into the extracellular fluid (5, 6). Assuming that adrenal hormone administration actually does produce a transfer of sodium, chloride and water into the extracellular fluid, it was considered likely that bone and dense connective tissue might provide a source of those electrolytes. Accordingly, the electrolyte and water composition of bone and tendon as well as muscle were measured in both control and hormone treated monkeys.

MATERIALS AND METHODS

These studies were performed on unanesthetized, adult, male cynomolgus monkeys. Female animals were not used in order to avoid the possible effects of hormonal changes upon water and electrolyte metabolism. Anesthetic agents were avoided since these have been shown to exert an effect upon the distribution of body fluids (7).

Pre-treatment control values for normal body weight, volume of distribution of inulin, inulin clearance (GFR), and plasma sodium, potassium and chloride concentrations were obtained in each monkey studied. Thereafter, each animal received 10 mg. of DCA intramuscularly daily for five days. Body weight, volume of distribution of inulin and plasma electrolyte concentrations were again determined. As soon as these measurements were completed, the animals were sacrificed and tissue samples of muscle, bone and tendon were obtained. Beginning five days before the pre-treatment studies, each monkey received a diet of one orange, three bananas, and distilled water *ad libitum* daily. On analysis, this diet contained 0.13 mEq. of sodium, 31.0 mEq. of potassium and 2.5 mEq. of chloride per day. The electrolyte and water content of the tissues of the control group were determined in untreated normal monkeys of comparable weight. The control animals were maintained on the same low salt diet as the hormone treated group for a period of eight to ten days before they were sacrificed.

The volume of distribution of inulin (fluid phase of the extracellular compartment, inulin space) was determined by the post-infusion technique (8, 9) wherein the plasma inulin concentration (corrected for blank) present at the end of an equilibrating infusion is divided into the amount of inulin excreted in the urine after a steady equilibrating infusion has been discontinued. Inulin clearances were performed by standard methods previously used in this laboratory.

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TABLE I

Body weight and inulin space before and after DCA administration

Monkey number	Weight		Inulin space	
	Kg.		*ml.*	%
25	5.8	Control	955	
	5.8	DCA	1216	
		Difference	+261	27
39	5.8	Control	908	
	5.5	DCA	1280	
		Difference	+372	41
44	7.4	Control	1214	
	6.9	DCA	1512	
		Difference	+298	25
45	5.1	Control	680	
	5.0	DCA	922	
		Difference	+242	35
46	4.1	Control	552	
	4.1	DCA	784	
		Difference	+232	42

The animals were sacrificed by decapitation in order to avoid the influences of anesthetic agents, exsanguination or prolonged agonal states. Immediately thereafter, tissue samples were obtained and cleaned of as much fat and connective tissue as possible. All marrow and periosteum were removed from bone specimens so that only the cortical portion remained. The samples were placed in weighing bottles, minced and weighed immediately. The tissues were then placed in a drying oven (100° to 105° C.) for 48 hours, by which time constancy of weight was attained. Bone required a longer period of drying (five days). The total water content of the tissues was calculated as the weight loss which resulted from drying. For sodium, potassium and chloride analysis, dried tissues in known amounts were placed in vitreosil crucibles and ashed over night at 450° to 500° C. in a muffle furnace. Dry samples to be analyzed for chloride were moistened with a saturated solution of sodium carbonate and those to be analyzed for sodium and potassium with concentrated sulfuric acid to prevent volatilization of chloride, and sodium and potassium, respectively. The tissues to be analyzed for chloride were ashed in a separate muffle furnace from those analyzed for sodium and potassium in order to prevent cross contamination. Ashed tissues were analyzed for chloride by the Van Slyke and Hiller modification of the Sendroy technique (10). The ashed tissues to be analyzed for sodium and potassium were dissolved in 1 N sulfuric acid and diluted with distilled water and appropriate equantities of lithium nitrate. The resulting solution was then analyzed for sodium and potassium in an internally standardized flame photometer. The fat content of bone and muscle was determined by the use of a soxhlet apparatus. Blood contamination of muscle was determined after the method of Eichelberger and Roma (11). That the calcium of bone does not interfere with the determination of sodium and potassium in a flame photometer was demonstrated by the use of an aqueous solution of sodium and potassium chloride in the concentration obtained in bone analysis. Duplicate aliquots were removed and calcium was added to one in an amount calculated to be the concentration present in solutions analyzed for bone sodium and potassium. The electrolyte concentrations in the various aliquots were not found to be significantly different. The electrolyte content of tendon is expressed as mEq. per Gm. of dry tissue, those of cortical bone as mEq. per Gm. of fat free, dry tissue, and the values in muscle as mEq. per Gm. of blood free, fat free, dry tissue. The water content of all tissues is expressed as Gm. per Gm. of wet tissue.

Plasma was analyzed for chloride by the Van Slyke and Hiller modification of the Sendroy technique (10). Sodium and potassium concentrations in plasma were determined by the use of an internally standardized flame photometer. Inulin was analyzed by the resorcinol method of Schreiner (12).

RESULTS

In five of the nine DCA treated monkeys studied, the body weight and volume of distribution of inulin were measured before and after DCA treatment (Table I). No significant change in body weight occurred as a result of the treatment. Each of the five animals studied presented significant expansion of the inulin space. Post-treatment volumes averaged 34 per cent (25

TABLE II

Plasma electrolyte concentrations before and after DCA administration

Monkey number		Na	K	Cl
			mEq./L.	
19	Control	141.8	3.3	111.7
	DCA	140.0	3.5	113.8
23	Control	142.0	3.6	114.3
	DCA	139.4	3.8	110.0
25	Control	140.8	4.4	117.3
	DCA	141.6	3.9	114.8
29	Control	139.0	3.6	107.5
	DCA	142.0	3.4	110.0
39	Control	142.0	3.0	109.1
	DCA	134.5	3.0	102.2
44	Control	135.0	3.1	105.0
	DCA	140.0	3.7	112.4
45	Control	138.0	4.2	102.8
	DCA	138.0	4.2	109.8
46	Control	134.2	4.0	108.4
	DCA	135.7	4.7	112.6

TABLE III

The values of water and electrolyte contents of muscle, bone and tendon of normal and DCA treated monkeys

	Monkey	Muscle†				Bone‡				Tendon§			
		Water*	Na	K	Cl	Water*	Na	K	Cl	Water*	Na	K	Cl
		Gm./Gm.	*mEq./Gm.*	*mEq./Gm.*	*mEq./Gm.*	*Gm./Gm.*	*mEq./Gm.*	*mEq./Gm.*	*mEq./Gm.*	*Gm./Gm.*	*mEq./Gm.*	*mEq./Gm.*	*mEq./Gm.*
	7	0.777	0.1037	0.4403	0.0712								
	8	0.763	0.1043	0.4330	0.0546								
	10	0.765	0.1175	0.4475	0.0733								
	11	0.779	0.1163	0.4544	0.0662								
	22	0.757	0.0950		0.0576	0.120	0.3235	0.0047	0.0227	0.576	0.2094	0.0208	0.2315
Normal	30	0.793				0.129	0.3110	0.0063	0.0250	0.583	0.2064	0.0278	
monkeys	34	0.765		0.4161	0.0651	0.122			0.0230	0.577			0.2274
	40	0.765	0.0907	0.4529	0.0609	0.125	0.3072	0.0062	0.0218	0.562	0.2280	0.0315	0.2226
	41	0.770		0.4398	0.0781	0.112	0.3100	0.0100	0.0206	0.556	0.2070	0.0328	0.2271
	43	0.783	0.1083	0.4610	0.0628	0.109	0.3000	0.0077	0.0246	0.599	0.2240	0.0308	0.2393
	48	0.790	0.0957			0.104	0.3200	0.0083	0.0203	0.587	0.2092	0.0203	0.2055
	49	0.759	0.0903			0.111	0.3068	0.0094	0.0211	0.585	0.2072	0.0215	0.2207
	Average	0.772	0.1023	0.4431	0.0655	0.117	0.3112	0.0075	0.0224	0.578	0.2130	0.0265	0.2250
	S.D.	0.011	0.0097	0.0133	0.0072	0.008	0.0075	0.0016	0.0016	0.012	0.0083	0.0050	0.0097
	19					0.117	0.3451	0.0121	0.0202	0.572	0.1902	0.0305	0.2052
	23	0.762	0.1171	0.4475	0.0721	0.116	0.3194	0.0075	0.0228	0.580	0.2087	0.0240	0.2058
	25	0.765	0.1096	0.4312	0.0732	0.115	0.3155	0.0131	0.0211	0.561	0.2321	0.0290	0.2314
DCA	29	0.753	0.1915	0.4709	0.1050	0.122	0.3305	0.0100	0.0192	0.570	0.1803	0.0152	0.1942
treated	39	0.743	0.1409	0.3527	0.0908	0.122	0.3200	0.0075	0.0246	0.564	0.2009	0 0222	0.2131
monkeys	44	0.755	0.1331	0.3613	0.0667	0.127	0.3217	0.0073	0.0211	0.569	0.2025	0.0304	0.2082
	45	0.764	0.1007	0.4036	0.0552	0.130	0.3138	0.0088	0.0200	0.586	0.1900	0.0224	0.2313
	46	0.760	0.1410	0.4134	0.0870	0.113	0.3090	0.0066	0.0222	0.587	0.2167	0.0285	0.2337
	50	0.765	0.1340	0.4832	0.1154	0.134	0.3057	0.0088	0.0210	0.573	0.1958	0.0258	0.2087
	Average	0.757	0.1338	0.4205	0.0837	0.122	0.3201	0.0091	0.0214	0.573	0.2020	0.0253	0.2175
	% change	−2.0	+30.8	−7.4	+27.8	+4.7	+2.1	+21.3	−4.4	−0.9	−5.1	−4.5	−3.3
	t	3.13	3.18	1.292	2.514	1.285	1.698	1.507	1.213	0.890	2.015	0.4575	1.156
	p	0.005	0.005	0.100	0.010	0.100	0.050	0.080	0.100	0.200	0.030	0.300	0.150

* In all tissues, water is expressed as Gm. per Gm. of wet tissue.
† Electrolytes are expressed as mEq. per Gm. of fat free, blood free, dry tissue.
‡ Electrolytes are expressed as mEq. per Gm. of fat free, dry cortical bone.
§ Electrolytes are expressed as mEq. per Gm. of dry tissue.

to 42 per cent) beyond those obtained prior to DCA administration. The plasma concentrations of sodium, potassium and chloride in eight monkeys were not significantly altered by the treatment (Table II).

A comparison of water, sodium, potassium and chloride contents of muscle in normal and DCA treated animals is presented in Table III. Only a small diminution occurred in water content. The 30 per cent rise in sodium content (0.03 mEq. per Gm.) in the treated group represents a considerable increment above the normal. This change was accompanied by a similar increase in chloride content of 0.018 mEq. per Gm. which averaged 27 per cent. The validity of these changes is attested to by t values of 3.18 and 2.514 and p values of 0.005 and 0.01 for sodium and chloride, respectively. The fall in potassium content averaged 0.04 mEq. However, significant changes occurred in only half of the animals. A comparison of the electrolyte and water composition in bone of normal and DCA treated monkeys revealed small changes of questionable significance (Table III). Similarly, the tendon in both groups showed no noteworthy difference (Table III).

No demonstrable change occurred in the glomerular filtration rate as a result of DCA administration.

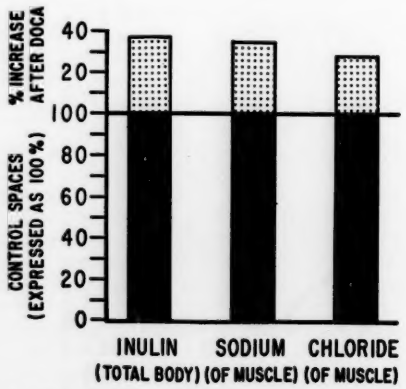

FIG. 1. THE EFFECT OF DCA ON FLUID AND ELECTROLYTE SPACES

The black portions of the bar graphs represent the total body inulin spaces and the calculated sodium and chloride spaces of muscle in the control animals expressed as 100 per cent. The stippled areas represent the per cent increase in those spaces following DCA administration.

DISCUSSION

The mean expansion of the inulin space of 34 per cent compared reasonably well with that noted after the administration of DCA, cortisone, and adrenocorticotropic hormone (ACTH) in dogs and man, respectively (1, 2). The 30 per cent increment in sodium and chloride content of muscle without any change in the plasma electrolyte composition suggests a comparable expansion in the extracellular fluid phase of muscle. The similarity between this expansion and that detected with the inulin space (Figure 1) argues that a true increase in extracellular volume had occurred. It is difficult to explain the increased quantity of salt in the muscle by any other hypothesis. The quantity of salt contained in the low salt diet throughout this five day period could account for only one-fourth of such an expansion. That the sodium content of muscle increased somewhat more than the chloride content may be attributed to the shift of a fraction of the retained sodium into cells to replace some of the lost potassium. These experiments, then, support the conclusion that the administration of DCA effects a transfer of sodium and chloride into the fluid phase of the extracellular compartment. The absence of comparable changes in bone and tendon electrolyte composition indicates that this redistributed electrolyte did not derive from these solid phases of the extracellular compartment. Some have suggested that the gastrointestinal tract might serve as a source of salt and water (1, 2), and these experiments indirectly suggest that such may be the case.

The slight fall in the total muscle water content suggests that the intracellular volume of muscle decreased after DCA administration. The outward shift of cell water may have occurred in association with the slight potassium loss and/or in order to maintain extracellular iso-osmolarity. The latter hypothesis would imply that the salt was transferred into the extracellular phase of muscle in relatively hypertonic concentrations.

The absence of a marked fall in muscle and plasma potassium and the meager degree of extracellular alkalosis are somewhat different from the typical responses described by others after DCA administration (13, 14). Most of the experimental work was performed in animals

treated with the hormone for prolonged periods and maintained on normal or even high salt intakes. It has been shown by others that DCA administration in animals or man maintained on salt-free diets causes far less urinary potassium loss (15). The meager degrees of potassium deficiency and extracellular alkalosis are, therefore, probably attributable to the marked salt restriction and the short period of hormonal treatment.

SUMMARY

1. The administration of DCA produced an increment in inulin space in monkeys maintained on a low salt diet.

2. Proportionate increases in the sodium and chloride spaces of muscle were also observed.

3. The sodium and chloride transferred to the extracellular fluid did not derive from bone or tendon.

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CORRECTION OF DEFECTS IN CLOTTING ACCELERATOR ACTIVITY BY ADMINISTRATION OF METHIONINE AND VITAMIN K AND OF A NEW SULFHYDRYL–SUBSTITUTED METHYLNAPHTHOQUINONE, VITAMIN K–S(II) [1, 2]

By JOHN R. CARTER AND E. D. WARNER

(From the Department of Pathology, State University of Iowa, Iowa City, Iowa)

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Recent studies on the role of sulfur-containing linkages in blood coagulation have contributed to the growing fund of knowledge of the biochemistry of prothrombin, its derivatives and activation (1–5). It is the purpose of this paper to present the salient data of a related series of investigations, extending over a nine-year period, which have resulted in 1) the discovery of a synergistic effect of DL-methionine and vitamin K on accelerator activity in the transformation of prothrombin to thrombin in both normal and pathological states; 2) the design, preparation, experimental and therapeutic trial of S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercaptopropionic acid, designated as vitamin K-S(II), a compound comparable in its effect on accelerator activity to the concomitant administration of methionine and vitamin K; and 3) the complete correction of both factor V and factor VII acquired deficiencies in two human cases and of the hemorrhagic manifestations associated with them by these compounds.

To minimize confusion, we have chosen to employ terminology and concepts in current usage with respect to the presumed separate identities of factor V and factor VII. Lewis and Ware (6) have challenged this assumption, presenting evidence that probably there is only one fundamental accelerator system in human plasma rather than two, and that changes in accelerator activity may result from alterations of the prothrombin molecule. The studies of Seegers, Alkjaersig, and Johnson (7), indicating that factor VII is a derivative of prothrombin (autoprothrombin I), and our own quantitative studies on –S–S– bonds common to purified bovine prothrombin and its derivatives, support the hypothesis that chemical alterations of a single complex glycoprotein molecule may exhibit prothrombin, thrombin or accelerator activity (4). The term "accelerator," as used in this paper, will refer to the total clotting accelerator activity of the plasma as measured by the method indicated below.

METHODS

Venous blood was collected in 1.85 per cent potassium oxalate in standard glassware using the two-syringe technique. The oxalated plasma was quick frozen and stored at −30° C. for at least one hour before being used in clotting studies. The thromboplastin used in all assays, except the prothrombin time, was a stable, potent, heat treated, centrifuged saline extract of beef lung which was free from prothrombin, thrombin and accelerator activity (8). Prothrombin concentration was estimated quantitatively by the modified two-stage method (9). 'Prothrombin activity' was determined by a one-stage method which consisted of incubating 0.1 ml. crude saline extract of beef lung thromboplastin, 0.1 ml. plasma and 0.1 ml. 0.025 M $CaCl_2$ solution at 37° C. Fibrinogen was determined by a semimicro-Kjeldahl method on the washed fibrin clot resulting from the addition of thrombin to the plasma.

Clotting accelerator activity was estimated quantitatively by our two-stage method (8). This method, which we have employed since 1950, utilizes three variables: onset of thrombin formation, conversion rate and thrombin yield. During the early years of this study, plasma treated with $HgCl_2$ was used as a source of accelerator-free plasma (10) and no distinction between factor V (Ac-globulin, labile factor, proaccelerin) and factor VII (stable factor, proconvertin, serum prothrombin conversion accelerator) was made. For the past five years, defibrinated oxalated human plasma, to which an additional amount of potassium oxalate is added (2 Gm. per 100 ml.) and which is aged 12 to 14 days at room temperature (11) or 5 to 6 days at 37° C., has been used for this purpose. Appropriately diluted in our test system, this preparation has contained 150 to 200 Iowa units per ml. of prothrombin and neither factor V nor factor VII activity has been detected. The results of accelerator assays using these two different substrates have been strictly comparable. Factor V and factor VII, specifically, were estimated quantitatively by this method also.

[1] Aided by a grant from Parke, Davis and Co.

[2] Presented before the Thirtieth Annual Meeting of the Central Society for Clinical Research, Chicago, November 1 and 2, 1957.

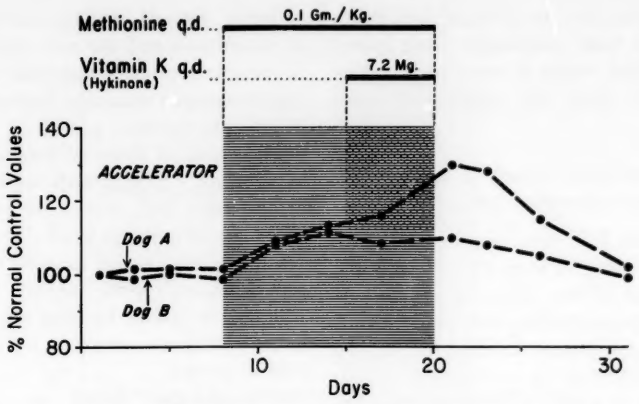

FIG. 1. DATA FROM NORMAL DOGS GIVEN DL-METHIONINE (DOG B) AND DL-METHIONINE PLUS VITAMIN K (DOG A)

Neither aged serum nor $BaSO_4$-adsorbed plasma when added separately to accelerator-free, prothrombin-rich plasma will elicit an accelerator response, but when both are added to the system, accelerator activity occurs equivalent to that of native whole plasma. Thus, both factor V and factor VII must be present to demonstrate accelerator activity by this method. This has been shown previously in test systems using highly purified clotting reagents (4). Prothrombin and factor VII-free plasma are prepared by mixing $BaSO_4$ with the oxalated plasma (100 mg. per ml.) constantly for 30 minutes at room temperature. Factor V-free serum is obtained by aging 48 hours (for human) and not less than 75 hours (for dog, rat) at room temperature. In all accelerator assays, accelerator-free substrate has been of human origin, but factor V and factor VII-free reagents have been species specific.

The standard stock diet for all dogs was Purina dog chow.

ANIMAL EXPERIMENTS

I. Historical

Preliminary experiments, begun in 1947,[3] were designed to test the hypothesis that the rate of regeneration of prothrombin following liver injury might be increased by the concomitant administration of vitamin K and DL-methionine. This was suggested by existing knowledge of the prothrombin-vitamin K relationship, the role of the quinones in oxidation-reduction systems, and especially the protective effect of methionine and cystine against chloroform injury in the protein depleted dog (12).

[3] U. S. Naval Medical School, Bethesda, Maryland.

It was observed that the rate of recovery of prothrombin activity (one-stage) in mature guinea-pigs, rendered hypoprothrombinemic by chloroform, was increased somewhat by the administration of either DL-methionine or vitamin K, but was decidedly more rapid when both methionine and vitamin K were given concomitantly. The prothrombin activity exceeded that of the control animals by as much as 70 per cent at the end of the recovery period. This was greatly in excess of the slight overcompensation generally observed in the normal (untreated) recovery period.

II. Effect of DL-*methionine and vitamin K administration in normal dogs*

Four normal healthy mongrel dogs (18 to 27 Kg.) were fed 0.1 Gm. per Kg. DL-methionine by mouth daily for from 12 to 14 days in addition to the stock diet. During the second half of this two-week period, they were given 7.2 mg. vitamin K (Hykinone®) by vein daily. A typical experiment is shown in Figure 1 (Dog A). The results were qualitatively similar in all four dogs. Prothrombin, fibrinogen and hematocrit values remained essentially unchanged. With the feeding of methionine, accelerator values gradually increased. When vitamin K was given concomitantly with the methionine, the increase became even greater. In three other dogs (*e.g.*, Figure 1, Dog B), in which methionine but not vitamin K was given, there was a gradual, mild rise in ac-

celerator values also, but the increase was never as great as when both compounds were given. There was a gradual return of accelerator values to normal levels when the compounds were stopped.

III. Effect of methionine vitamin K and vitamin K-S(II) in cholecystnephrostomized dogs

Bile-renal fistulas (13) were produced successfully in six adult mongrel dogs (15 to 16 Kg.). It is well recognized that diversion of the bile through the kidney results in a bleeding tendency (14) caused by hypoprothrombinemia (15) which results from a deficiency of, and which is corrected by, vitamin K (16). Except where otherwise indicated, the dogs were maintained on the stock diet. Approximately every six weeks, vitamins A and D were given parenterally. A variety of experiments was performed on each of these dogs in duplicate or triplicate and the period of study in each dog covered not less than one year. During this period, the dogs gradually lost weight and became anemic. Some spontaneously hemorrhaged and several developed decubitus ulcers. These complications were treated symptomatically by transfusions, antibiotics, and so forth. One of the dogs died during the period of observation and the rest ultimately succumbed at variable periods of time after one year. Three dogs developed muscular dystrophy. The values of the preoperative period of the experimental dogs as well as those of normal dogs were used as controls. Experiments were begun after the prothrombin and accelerator values had fallen below the 50 per cent level. The time required to reach this level varied from 80 to 130 days. Except when on specific therapy, there was a slow progressive decline in these clotting values until, at the end of one year, they were from 5 to 20 per cent of normal.

DL-*methionine added to protein-free diet.* Three bile-renal fistula dogs, maintained on the stock diet for approximately 175 days, were placed on the following protein-free diet: 327 Gm. sucrose, 450 Gm. starch, 20 Gm. salt mixture, 200 Gm. Crisco, 1.5 Gm. L-tryptophan, 1.5 Gm. L-cystine, and 2 tablespoons polyvitamin dispersion; total, 1,000 Gm. The dogs were offered 500 Gm. per day for 21 days and consumed an average of 85 per cent of the diet per day. The results were similar and those of one of the dogs are shown in Figure 2. There was a pronounced

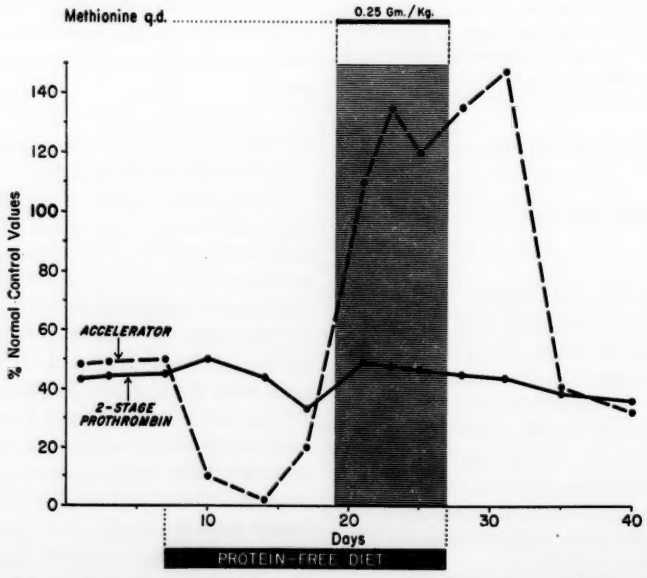

FIG. 2. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS ON PROTEIN-FREE DIET AND PROTEIN-FREE DIET SUPPLEMENTED WITH DL-METHIONINE

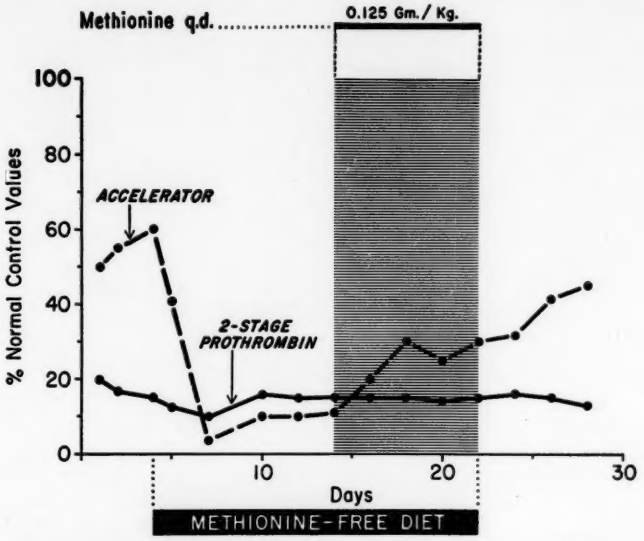

FIG. 3. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS ON DL-METHIONINE-FREE DIET AND DL-METHIONINE-FREE DIET SUPPLEMENTED WITH DL-METHIONINE

decrease in accelerator values on this diet. Two-stage prothrombin and fibrinogen values remained essentially unchanged. With the addition of DL-methionine to the diet (4 Gm. per day for nine days; 0.25 Gm. per Kg.), accelerator values increased rapidly and exceeded those of the control animals. There was a concomitant mild increase of fibrinogen of questionable significance, but again prothrombin values were not affected. With the return of the dogs to the stock diet, accelerator values dropped to the pretreatment level by the eighth postmethionine feeding day. The elevated accelerator activity, which lasted for at least four days after the methionine feeding was stopped, suggested a limited degree of storage. No significant change in hematocrit levels was noted. One-stage prothrombin values tended to parallel those of the accelerator, but the magnitude of change was considerably less.

DL-methionine added to methionine-free diet. After a period of 50 days on the stock diet, two of the same three dogs were placed on the following diet: 24 Gm. methionine-free casein hydrolysate (1,000 ml.), 0.75 Gm. L-cystine, 0.75 Gm. DL-tryptophan, 139 Gm. sucrose, 225 Gm. starch, 10 Gm. salt mixture, 100 Gm. Crisco, and 1 tablespoon polyvitamin dispersion. The dogs were offered 500 Gm. per day for 19 days and consumed an average of 95 per cent of the diet per day. The casein hydrolysate was rendered free from methionine and tryptophan by the method of Albanese (17). The results were similar, and those of one of the dogs are shown in Figure 3. By the fourth day on this diet, accelerator values had fallen from a level of from 50 to 60 per cent to from 3 to 10 per cent of normal, and they remained at this low level for the next eight days. Fibrinogen remained at essentially normal levels; one- and two-stage prothrombin values remained low throughout the test period. With the addition of 0.125 Gm. per Kg. DL-methionine to the diet for nine days, half as much as that given in the previous experiment, there was a moderate, gradual increase of about 20 per cent in accelerator values. Fibrinogen and one- and two-stage prothrombin values remained essentially unaltered. After the return to the stock diet, accelerator values continued to rise slowly. Hematocrit levels were not affected.

DL-methionine added to methionine-free diet in normal dogs. As a corollary experiment to that just preceding, two normal dogs which had been

maintained on the stock diet for two years were placed for 14 days on the methionine-free diet. The dogs consumed an average of 70 per cent of the diet per day, occasionally vomited and were force fed. Both dogs showed similar changes in the clotting factors measured; the results on one of the dogs are shown in Figure 4. Within two days on this diet, the values for accelerator, one-stage prothrombin, and fibrinogen began to fall and by the eleventh day they had become reasonably stabilized at moderately low levels. Accelerator activity, for example, reached 40 per cent of normal. The diet was continued for an additional four days, but during these four days, beginning on the eleventh day and continuing for 13 days, the dogs also were fed 2 Gm. DL-methionine per day (0.125 Gm. per Kg. per day). They then were returned to the stock diet. With the addition of the methionine to the methionine-free diet, the values for these clotting tests promptly returned to essentially normal levels and so remained throughout the methionine supplemented stock diet feeding period. Again, the accelerator response was greatest, the values exceeding those of the normal by about 10 per cent during the period on the methionine supplemented stock diet. Throughout the test period, the two-stage prothrombin and hematocrit values remained unaltered.

Effect of vitamin K, methionine, and vitamin K plus methionine. In order to better evaluate the synergistic effect of vitamin K and methionine on accelerator activity, as suggested by earlier experiments, studies were made on three cholecystnephrostomized dogs, in duplicate. Before and during the experiments the dogs were maintained on the stock diet. None of the animals were considered critically ill and all lived for an additional three to four months. Because of the inherent biological and clinical variations in bile-renal fistula dogs, the quantitative results varied considerably, but in all six experiments, a significant reproducible qualitative trend was observed. The values in the six experiments were averaged, and are shown as a composite graph in Figure 5.

The intravenous injection of from 0.17 ml. (equal to 1.0 mg. anhydrous menadione bisulfite) to 1.0 ml. Hykinone® resulted in the expected elevation of prothrombin concentration in all dogs to normal levels. This vitamin K effect often lasted from four to six days, and the subsequent decline of prothrombin was slowly progressive. The accelerator response to vitamin K was slight, seldom greater than a 20 per cent increase, but again rather long-lasting in a few of the experiments. Methionine (4 Gm. per day; 0.25 Gm. per Kg. per day) was then fed for eight days prior to the second injection of vitamin K. This resulted in

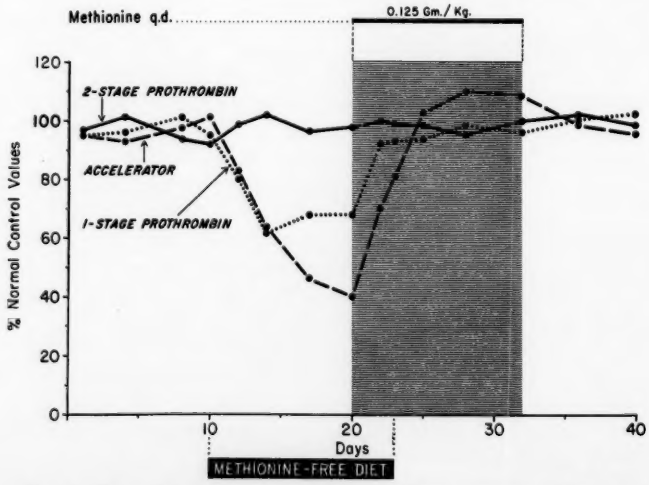

FIG. 4. DATA FROM NORMAL DOGS ON DL-METHIONINE-FREE DIET, DL-METHIONINE-FREE DIET SUPPLEMENTED WITH DL-METHIONINE AND STOCK DIET SUPPLEMENTED WITH DL-METHIONINE

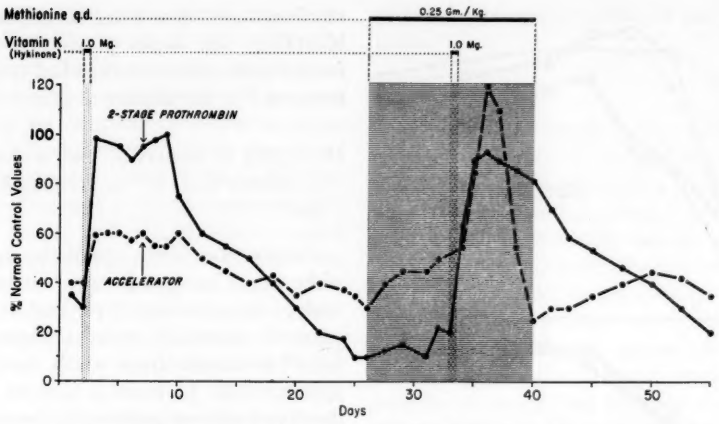

FIG. 5. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS GIVEN VITAMIN K, DL-METHIONINE AND VITAMIN K PLUS DL-METHIONINE

an average increase of 20 per cent in accelerator activity but an essentially unaltered prothrombin concentration. The methionine feeding was continued for an additional eight days. On the eighth day of the 16-day methionine feeding period, Hykinone® again was given intravenously in the same dosage as before. Prothrombin levels again rose and were comparable to those produced by the first injection. Accelerator values in all dogs, in contrast to those obtained when vitamin K alone was given, promptly returned to normal or above normal levels. In spite of continued methionine feeding, the levels were not sustained when the vitamin K was no longer operative. Thus, the biological synergistic effect of DL-methionine and vitamin K on accelerator activity again was demonstrated.

Effect of vitamin K-S(II). On the basis of the preceding experiments, it seemed reasonable to suppose that a sulfhydryl-substituted methylnaphthoquinone might exert a similar effect on accelerator activity. S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercaptopropionic acid, designated as vitamin K-S(II), was prepared.[4] Although slightly modified by one of us (J.R.C.) to realize better yields, the method of preparation of vitamin K-S(II) has been described by Hanna (18). The compound was dissolved in 1.0 per cent $NaHCO_3$ (1 mg. per ml. concentration), adjusted to pH 7.4, passed through a sintered glass filter and administered intravenously to 4 bile-renal fistula dogs on 10 comparable occasions. The animals were maintained on the stock diet for at least one month prior to each test period of administration of the drug. The results were similar in all

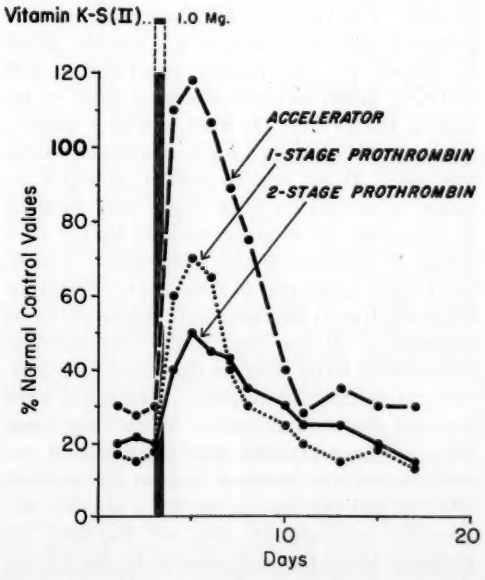

FIG. 6. DATA FROM CHOLECYSTNEPHROSTOMIZED DOGS GIVEN A SINGLE DOSE OF VITAMIN K-S(II)

[4] We are indebted to Dr. Calvin Hanna, formerly of the Department of Pharmacology, for the original preparation of vitamin K-S(II).

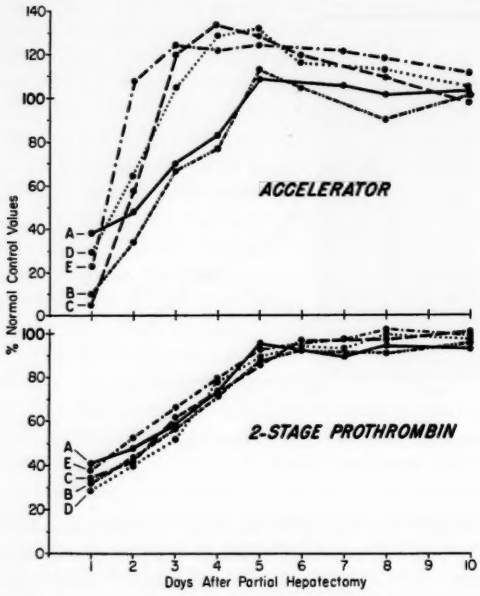

FIG. 7. DATA FROM PARTIALLY HEPATECTOMIZED RATS ON FIVE DIETARY REGIMENS

Curves: A, Controls; B, Vitamin K; C, DL-Methionine; D, Vitamin K plus DL-Methionine; E, Vitamin K-S(II).

10 tests. The values in the 10 experiments were averaged, and are shown as a composite graph in Figure 6. In control experiments with $NaHCO_3$ alone, no detectable alterations of the clotting factors were observed. With a single 1 mg. dose (0.07 mg. per Kg.), accelerator values, previously 30 per cent of normal, promptly returned to normal levels or above, thus equalling or exceeding the response effected by the same dosage of Hykinone® plus methionine. The effect usually lasted two to three days. Prothrombin concentration increased more slowly and never exceeded a 30 per cent rise. The one-stage prothrombin levels reflected the combined accelerator and prothrombin response. Fibrinogen levels were not altered significantly. When 5 mg. quantities of the compound were administered, accelerator activity increased to even higher levels (150 to 160 per cent of normal), but the prothrombin concentration was not increased appreciably above the level effected by the 1.0 mg. dosage. Accelerator and prothrombin values, 10 to 15 per cent of normal, in two dogs in near moribund condition, failed to respond to vitamin K-S(II). Six weeks previously, these clotting factors in the same two dogs had responded to the compound in the manner indicated above.

IV. Effect of vitamin K and/or methionine, and of vitamin K-S(II) on partially hepatectomized rats

Twenty-two adult, partially hepatectomized white rats (Sprague-Dawley) were used in this study. An estimated 70 per cent of the liver was removed aseptically under intraperitoneal Nembutal® anesthesia from which they recovered in approximately 12 hours. None of the rats used developed obvious pulmonary, liver or wound infections. They were offered only water and 20 per cent glucose for the first 24 hours after operation. Beginning with the second 24 hours and continuing throughout the experimental period, they were offered water and the specific diets, as indicated below, which they consumed *ad libitum*. The animals were divided into five dietary groups, the constituent common to all of which was ground Rockland rat ration, adequate in vitamin K. This was supplemented as follows:

A. Controls, five rats; no supplement.
B. Vitamin K, four rats; 1 mg. menadione per Gm. of diet.
C. Methionine, five rats; 1 Gm. DL-methionine per 50 Gm. of diet (2 per cent).
D. Vitamin K plus methionine, three rats; 0.5 mg. menadione per Gm. of diet plus 1 Gm. DL-methionine per 50 Gm. of diet (2 per cent).
E. Vitamin K-S(II), five rats; 1 mg. vitamin K-S(II) per Gm. of diet (three rats); 1 mg. vitamin K-S(II) per Gm. of diet plus 1 ml. (1 mg. per ml. concentration, dissolved in 1 per cent $NaHCO_3$) vitamin K-S(II), daily, intraperitoneally (two rats).

The average values of two-stage prothrombin and accelerator determinations, respectively, in each group of rats during the 10 to 20 day experimental period are shown in Figure 7. With respect to accelerator activity, the recovery rates of the control (curve A) and vitamin K (curve B) groups of rats were similar, approximately 20 per cent of the normal activity per day. Normal values were not obtained until the fifth postopera-

tive day. The recovery rates of the methionine (curve C) and of the vitamin K plus methionine (curve D) groups also were similar, but on these diets the rates were approximately 45 per cent per day, the values returning to normal or above on the third postoperative day. Even more rapid and impressive was the recovery rate of the vitamin K-S(II) (curve E) group, approximately 85 per cent in one day with normal values or above achieved by the second postoperative day, the rats having been on the diet for only 24 hours. The results were essentially the same whether the vitamin K-S(II) was given orally or intraperitoneally and orally. Two of the rats of this group were given 50 mg. vitamin K-S(II) by stomach tube seven days after the accelerator values had returned to normal. There was no additional effect on either accelerator or prothrombin activity.

It is also apparent from Figure 7 that rats on the diets supplemented with methionine and vitamin K-S(II) exhibited rather pronounced hyperaccelerator activity following the recovery phase as compared with that of the control and menadione groups. The overcompensation persisted for 10 days or longer. Factor V and factor VII, specifically, were estimated routinely following partial hepatectomy and until recovery was complete. Barium sulfate-adsorbed plasma and aged serum added separately to the accelerator test system each resulted in only partial correction of the total accelerator activity. The aged serum, however, did effect a decidedly greater correction than did the $BaSO_4$-adsorbed plasma, thus indicating a greater deficiency of factor VII than of factor V. When both reagents were added together to the test system, complete correction was achieved.

The recovery rates of prothrombin concentration (two-stage) in all groups of rats were essentially similar, approximately 15 per cent per day for the first five postoperative days. At the end of this period, the levels were still only 90 per cent of normal, an additional 5 to 10 days elapsing before recovery was complete regardless of diet. Thus, the effect of the dietary supplements on prothrombin concentration, in striking contrast to that on accelerator, was nil. One-stage prothrombin values, routinely determined in all groups of rats throughout the experiment, closely paralleled accelerator rather than prothrombin values with respect to rate of recovery time required for complete restitution and overcompensation.

V. Toxicity studies

Eight healthy mongrel dogs (12 to 16 Kg.) were the recipients, on repeated occasions, of vitamin K-S(II) in doses varying from 100 to 1,000 mg. daily, by mouth, for from 7 to 30 days. The dogs remained clinically healthy at all times. Two dogs were sacrificed after courses of this drug. Grossly and microscopically, no significant anatomical abnormalities were found. Similar studies were made on six normal albino rats, none of which exhibited evidence of toxicity. Clinical evidence of toxicity has not been observed in human patients who have received vitamin K-S(II) orally in doses varying from 50 to 500 mg. daily for from 2 days to 1 year, or intravenously, 20 mg. daily for 10 days.

CLINICAL STUDIES

Results of experimental work had progressed sufficiently by 1950 to suggest application to a man critically ill with a severe hemorrhagic diathesis, refractory to all the usual therapeutic measures. On repeated occasions, low accelerator values were normalized promptly by the concomitant administration of methionine and vitamin K and later by vitamin K-S(II). Clinical improvement with cessation of bleeding was noted on each occasion in spite of a persistent severe hypoprothrombinemia. Because of the complexity of the hemorrhagic disorder which has been studied extensively for six years, only those features relevant to the preceding experimental study will be presented in detail at this time. A second patient is the third recorded example of hypoprothrombinemia and "hypoproconvertinemia" during pregnancy (19, 20), but she had, in addition, a deficiency of factor V. The respective factors were overcorrected by vitamin K-S(II).

Case 1

C. M., a 57 year old male Caucasian house painter, was admitted to the medical service of the University Hospitals July 17, 1950, *in extremis*. Fatigue, weakness and general malaise had been noted for three months. He had received seven transfusions for severe anemia at another hospital, but two days following the last transfusion, had developed hematomata of the neck and jaw, the right leg and knee and palm of the left hand. Pro-

thrombin was reported to have been 12 per cent; after 300 mg. Hykinone®, the value was 19 per cent. The first evidence of a hemorrhagic tendency had occurred two years before when he bled profusely for 135 hours following a tooth extraction and had noted increased ease of bruising. He was wounded in World War I and had had numerous traumatic episodes, including other tooth extractions, over the years without unusual blood loss. There was no history of any bleeding tendency in his parents, grandparents, siblings, children or other blood relatives. He had never been jaundiced.

One-fourth of the oropharynx was occupied by a bulging submucosal hematoma. Other large hematomata were present in the right peritonsillar area, beneath the right sternocleidomastoid muscle, in both antecubital spaces, on both the dorsal and ventral surfaces of the left hand, and in the subcutaneous tissues of the right thigh. All had occurred spontaneously.

Laboratory studies. The patient was hospitalized until November 8, 1950, a total of 115 hospital days. Throughout this period, on subsequent hospital admissions, and at numerous intervals over the six year period, laboratory studies were made repeatedly. The following were usually *abnormal:* platelets, 60,000 to 100,000 per cu. mm.; clot retraction, none to partial at four hours; coagulation time (Lee-White), 35 plus minutes; serum calcium, 8.0 to 10.6 mg. per 100 ml.; cephalin cholesterol flocculation, 2 plus to 4 plus at 24 and 48 hours. Kolmer, Kline and VDRL tests were positive in 1:2 dilution. He emphatically denied venereal disease, and in view of the low titer and the blood dyscrasia, these were considered false positive reactions.

The following analyses were performed repeatedly: one-stage prothrombin; two-stage prothrombin; clotting accelerator; factor V and factor VII; fibrinogen; circulating anticoagulant (21); heparin (22); protamine titration (23); fibrinolysin (24).

Hospital course, therapy and results. As indicated in Figure 8, large doses of vitamins K (Hykinone®) and K_1, given intravenously, were unassociated with sig-

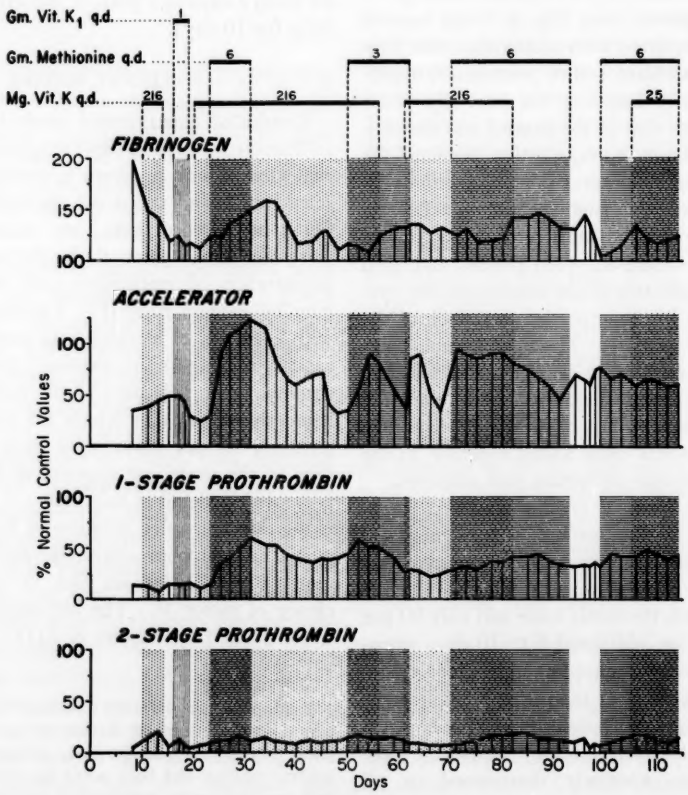

FIG. 8. DATA FROM PATIENT (CASE 1)

Note the accelerator response to the concomitant administration of DL-methionine plus vitamin K.

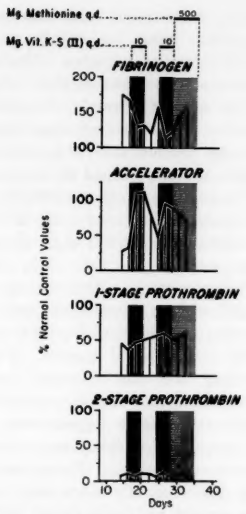

FIG. 9. DATA FROM PATIENT (CASE 1)

Note the accelerator response to intravenous administration of vitamin K-S(II).

nificant changes in prothrombin or accelerator concentration, and bleeding phenomena continued unabated. When, in addition to Hykinone®, DL-methionine was administered orally on the twenty-third day, *a prompt and spectacular rise in accelerator values and a significant elevation of one-stage prothrombin values* resulted. During the remainder of the patient's hospital stay, accelerator repeatedly responded to and could be maintained at near normal levels by the administration of vitamin K plus methionine, but neither compound alone was effective. Usually, a concomitant but decidedly less conspicuous response in one-stage prothrombin activity was obtained. The severe hypoprothrombinemia (two-stage) remained unaltered by the therapy. The initial apparent response of the hyperfibrinogenemia to the vitamin K compounds alone was not as evident in the later trials. Clinically, there was excellent correlation between absence of bleeding, resolution of hematomata and high accelerator levels. At no time, even when accelerator values were normal, was the coagulation time less than 35 minutes and clot retraction was never complete at four hours.

On the seventy-eighth and eighty-seventh days, 800 and 500 ml., respectively, of whole blood were given. These transfusions, some of which were with fresh blood, corrected the progressive hypochromic anemia but were without demonstrable effect on the clotting factors. Protamine (50 mg.) was given on the ninety-sixth day. No appreciable change in clotting factors was noted except that for the next four days fibrinogen values decreased appreciably. Ten days prior to discharge, he was placed on oral vitamin K (Synkayvite®) in addition to the methionine begun six days before. No significant alteration of clotting factors was observed. Lack of accelerator response may have been the result of too small a dose or lack of absorption or utilization of the orally administered vitamin K. He was discharged, greatly improved, on this regimen plus a high methionine diet (milk, meat and eggs).

Progress report. The patient was seen on five occasions at regular intervals from January, 1951, to June, 1952. Methionine and vitamin K were discontinued after January 21, 1951. Throughout this 17 month period, he had remained in good health and free from bleeding episodes. There was a progressive decline in accelerator levels to 30 per cent, with a concomitant rise in fibrinogen from 125 to 215 per cent. Other laboratory values were as stated initially.

Second hospital admission. The patient was readmitted on August 12, 1952, because of a large hematoma of the right elbow area. Several days previously he had noted gross hematuria and blood streaked stools. He appeared chronically ill, anemic, and had lost weight.

Because of the beneficial effect of vitamin K-S(II) on accelerator activity in the cholecystnephrostomized dogs, this compound was dissolved in 1 per cent $NaHCO_3$ (1 mg. per ml. concentration), adjusted to pH 7.4, passed through a sintered glass filter and administered to the patient, intravenously, in dosages and with results shown in Figure 9. Prothrombin concentration was not altered. Ten mg. dosages, on two occasions, promptly normalized the accelerator values. One-stage prothrombin activity gradually increased; fibrinogen, reciprocal with accelerator activity, decreased on vitamin K-S(II) therapy. No toxic reaction was noted. Bleeding (skin, urine, stools) stopped after the administration of vitamin K-S(II). On the thirty-fourth day, he was transfused and discharged on 500 mg. DL-methionine per day and a high caloric diet. He returned one month later. He had felt well, gained weight and had had no bleeding. Laboratory tests were virtually the same as they had been at the time of discharge. He was continued on 500 mg. DL-methionine per day.

Third hospital admission. Two weeks prior to this admission on January 14, 1953, the patient noted increasing fatigability, dyspnea, palpitation, pale complexion, decreased appetite, hematuria and ecchymoses. He had remained on the methionine, but this was stopped on admission. Other than those of the blood and blood clotting tests, the values of repeated laboratory procedures were similar to those given previously. Two blood transfusions (1,000 ml.) which partially corrected the severe hypochromic anemia were given on the third day after admission. These did not alter the low levels of prothrombin and accelerator activity.

On four occasions, vitamin K-S(II) was given orally in dosages and with results indicated in Figure 10. During the administration of the drug, accelerator values were within normal limits; when it was stopped, they returned to the pretreatment range (25 to 35 per cent). The response was prompt and cessation of it equally so, the latter indicating no significant storage or accumulative effect. One-stage prothrombin activity tended to

parallel accelerator activity, but the magnitude of the fluctuations was minimal by comparison. The lack of a more striking response may be accounted for by the exceptionally low, consistently unaffected prothrombin concentration which probably was not the result of a vitamin K deficiency. Fibrinogen values were consistently above normal, but not as high as they had been. No inverse relationship with accelerator activity occurred as had before. During the week prior to discharge, the patient was given six transfusions (3,000 ml.) of whole blood. He appeared clinically improved and was discharged on the one hundred first day to receive 500 mg. vitamin K-S(II), orally, every other day.

Progress report. The patient returned in May, June, and September, 1953. He had felt entirely well and had had no bleeding. Physical examinations were essentially negative. The red cell count and hemoglobin fell very slowly over this period. Other laboratory tests remained essentially unchanged. Accelerator activity remained normal or above; all other clotting factors were as before. Vitamin K-S(II), orally (500 mg. every other day), was continued.

Fourth hospital admission. The patient was readmitted on March 4, 1954, complaining of dyspnea, easy fatigability and weakness of legs which had begun insidiously three weeks before. Prior to this he had felt well and had had no bleeding. He had been taking vitamin K-S(II) as directed until one week prior to admission at which time his supply was exhausted. Physical examination was negative. Moderately severe hypochromic anemia was demonstrated. Accelerator activity was 30 per cent of normal. All other laboratory tests were similar to what they had been. He was started on 500 mg. vitamin K-S(II), orally, every other day. Accelerator values returned to normal in 24 hours, and remained normal; no other clotting factors were affected. The anemia was corrected with transfusions and he was discharged on 100 mg. vitamin K-S(II) every third day.

Fifth hospital admission. The patient remained in good health and without complaints until three weeks prior to admission on December 29, 1954, when he again noted signs and symptoms of anemia. He had experienced no bleeding and had tolerated the drug well. Physical examination was again essentially negative. Again, a moderately severe hypochromic anemia was demonstrated, but all other laboratory tests showed the same abnormalities as before. There was no blood in stools or urine and clinically there was no evidence of bleeding. The patient had been taking 100 mg. vitamin K-S(II) every third day as directed, and his admission accelerator level was 34 per cent. When the dosage was increased to 500 mg. per day, accelerator activity promptly rose to normal levels (95 to 110 per cent) and

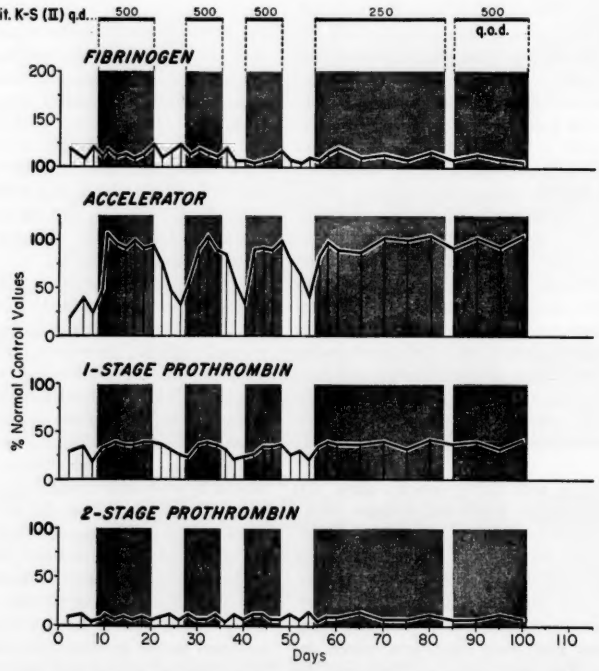

FIG. 10. DATA FROM PATIENT (CASE 1)
Note the accelerator response to oral administration of vitamin K-S(II).

there remained until the drug was stopped one month later (February 4, 1955). Other clotting factors remained at abnormal levels as before.

Progress report. The patient was checked nine times between February, 1955, and December, 1956. He had remained symptomatically well, had had no bleeding and was on no medication except iron salts. Accelerator values ranged from 80 to 100 per cent. He had transfusions in April, 1955, and in August, 1956, to correct his anemia. All other laboratory tests remained essentially the same as on previous admissions. He was seen again in April, 1957, with manifestations of anemia but with no history of recent bleeding episodes. On two separate occasions, low accelerator values (30 and 40 per cent) returned to normal levels following the oral administration of 300 mg. vitamin K-S(II) per day for two days.

Throughout the periods of therapeutic trial with vitamin K-S(II), the dosage varied from 10 mg. intravenously, to 500 mg. orally, daily, both quantities of which resulted in complete correction of low accelerator levels. The minimum effective oral dosage in this patient has appeared to be 300 mg. daily. The difference in magnitude between the oral and intravenous administration may have many explanations, one of which may be poor absorption of the drug from the gastrointestinal tract.

Factor V and factor VII activity. Numerous samples of the patient's plasma, both fresh and frozen (− 30° C.) throughout the six year period, were studied for factor V and factor VII activity. Low accelerator values always could be normalized by the addition of 0.03 ml. normal human $BaSO_4$-adsorbed plasma to the clotting system, whereas a similar quantity of normal 48 hour old human serum never corrected the deficiency, even partially. These results, therefore, indicate a deficiency of factor V, not factor VII, and further indicate that the corrective effect of vitamin K-S(II), as well as of methionine and vitamin K, was on factor V.

Additional studies. Details of the following features of this case will be published separately, but are summarized briefly here. The hypochromic anemia, most marked during the third and fifth admissions, was found to be associated with a moderately severe hemolytic mechanism on both occasions. The hemolytic anemia was considered to be another facet of his hemorrhagic disorder and not related to the therapeutic agents employed. Platelet agglutination studies in February, 1956, suggested the presence of both iso- and autoagglutinins.

An anticoagulant in plasma, but not in aged serum, could be demonstrated easily and in high titer for months at a time, but for other equally long periods none was detected or it was of such low titer as to be equivocal. No correlation between anticoagulant titer and other specific clotting factors, clinical manifestations, diet and therapy could be demonstrated. Using two-stage systems, neither the patient's plasma nor serum altered the prothrombin or accelerator concentration of normal plasma, nor did it alter the reaction rates in these assays. Antithrombin levels were always within normal limits, and no fibrinolysins were ever detected. No evidence could be obtained which would suggest a species specific antithromboplastin in the patient's plasma. Plasma heparin levels were within normal range and protamine titrations indicated no excess endogenous heparin or heparin-like substances in the patient's blood.

Case 2.

M.S., a 22 year old white married woman, one month pregnant, was admitted to the otolaryngology service on February 4, 1955, because of severe epistaxes. Numerous bleeding points were found in both nostrils, and, in spite of the application of both anterior and posterior nasal packs, the bleeding continued. Except for occasional epistaxes at age 11, the patient had never experienced abnormal bleeding or bruising previously. Menstrual blood loss always had been normal. The birth of her first child in 1952 was uneventful; the delivery had been accompanied by a total loss of 325 ml. of blood. There was no familial history of a bleeding tendency. The patient had been in excellent health and there had been no injury to the nose. Initial laboratory studies revealed the following: erythrocytes, 2.88 millions per cu. mm.; hemoglobin, 8.8 Gm. per 100 ml.; hematocrit, 23 per cent; coagulation time, 8 minutes; bleeding time, 6 minutes; platelets, 274,000 per cu. mm.; normal clot retraction; urinalysis, normal. A direct Coombs test was negative and serum bilirubin was 0.3 mg. per 100 ml.

From February 4 to 7, the patient received seven transfusions (3,500 ml. of banked blood) but the bleeding continued, at times profusely. Subsequently, massive nasal hemorrhage occurred, necessitating ligation of the right external carotid artery. On February 7 (fourth hospital day), blood clotting studies were requested; the significant abnormalities are shown in Figure 11. Accelerator, one-stage and two-stage prothrombin values were 10, 30 and 70 per cent, respectively, in spite of the numerous transfusions the patient had received. Prothrombin utilization was decreased. Fibrinogen, antithrombin, clotting time and clot retraction values were normal. There was no evidence of a circulating anticoagulant.

Vitamin K-S(II) (440 mg.) was given by mouth. The following day, after blood had been obtained for clotting tests, an additional 550 mg. was given. The compound was administered in divided dosages and was well tolerated. No transfusions or other drugs were given at this time. The effect was prompt and dramatic; all values had returned to normal levels within a few hours after the last dose of vitamin K-S(II). There was a rather pronounced and sustained hyperaccelerator activity after the drug was discontinued. A similar, but transient and less marked elevation of prothrombin concentration occurred. Prothrombin utilization returned to normal and all other clotting tests remained so. Nasal bleeding stopped completely and never recurred, even when the nasal packs were removed on the seventh hospital day. The patient was seen on six occasions from the time of discharge until May 17, 1955. Clotting tests were normal on each occasion. In December, 1955, she was delivered of her second child without excessive

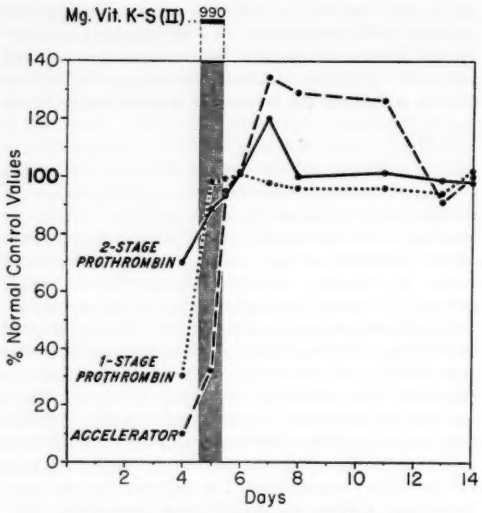

FIG. 11. DATA FROM PATIENT (CASE 2)

Note the accelerator response to oral administration of vitamin K-S(II).

bleeding, and remained in excellent health and free from abnormal bleeding through December, 1956.

Factor V and factor VII activity. Prior to treatment, the accelerator value was 10 per cent of normal. When 0.03 ml. of normal 48 hour old human serum was added to the system, there was a correction to 79 per cent. When 0.03 ml. of normal $BaSO_4$-adsorbed human plasma was added, there was a correction to 48 per cent. When both were added, the result was 96 per cent, indicating that the patient's plasma was noninhibitory to either heterologous factor V or factor VII. Thus, in this patient, a deficiency of both factor V and factor VII, predominantly the latter, existed. Both deficiencies presumably were corrected by vitamin K-S(II)—presumably, because spontaneous recovery, although unlikely, cannot be excluded.

DISCUSSION

The experimental data, accumulated from different animal species and by a variety of techniques, provide convincing evidence that accelerator levels may be increased by the synergistic action of DL-methionine and vitamin K and by vitamin K-S(II). Certain apparent incongruities regarding the accelerator response to methionine in the cholecystnephrostomized dogs deserve comment, however. The accelerator values fell equally when the dogs were on the protein-free (Figure 2) and the methionine-free (Figure 3) diets, but the response to the administered methionine was much more dramatic in the dogs on the protein-free diet. This could have resulted from the difference in the quantity of methionine fed or from the poorer physical condition of the dogs. The accelerator response to methionine alone, prior to the administration of vitamin K (Figure 5), also was by no means comparable to that observed in dogs on the protein-free diet (Figure 2). Since the quantity of methionine administered was the same in these experiments, an explanation for the difference might be the different diets. The stress impact of cholecystnephrostomy with its attendant complications is obviously unphysiological and cannot be ignored in attempting to evaluate these observations. Factor V and factor VII, specifically, were not determined in our bile-renal fistula series of experiments since factor VII was not sufficiently characterized at the time they were conducted. From the work of others (25, 26), we assume that the low accelerator levels in our cholecystnephrostomized dogs were a reflection of factor VII deficiency and that factor V values were presumably normal.

In contrast to the striking response of accelerator, neither fibrinogen nor prothrombin responded to methionine, alone or in combination with vitamin K, in any of the animal experiments except, of course, for the expected prothrombin response to vitamin K in the cholecystnephrostomized dogs. Prothrombin and fibrinogen were unaffected by either an excess or a deficiency of methionine. This suggests that, for short periods of time, exogenous sources of this amino acid are not essential to maintain prothrombin and fibrinogen levels in spite of the fact that amino acid analyses reveal significant quantities of methionine in both (27, 28). One-stage prothrombin activity tended to parallel that of accelerator except when the two-stage prothrombin level was particularly low, in which case the one-stage prothrombin activity usually was depressed.

It is apparent from the clinical data that the simultaneous administration of vitamin K and DL-methionine, and of vitamin K-S(II), had a sustained beneficial effect on accelerator activity. The first case, in addition to the therapeutic aspects, is, in itself, enigmatic and multifacetted. After six years of study, the etiology and pathogenesis are still obscure. Although no blood relatives were available for study, the disorder ap-

parently was acquired and unrelated to obstructive jaundice, severe liver disease, antibiotics, drug sensitivity, lead poisoning, sprue or celiac disease. In parenchymatous liver disease and vitamin K deficiency, both prothrombin and factor VII are decreased, indicating that both substances are formed in the liver and that both require vitamin K for synthesis (29). In this case, factor VII values have been consistently normal and neither vitamins K or K_1 in large doses corrected the clotting abnormalities. The dietary intake was always satisfactory.

The discrepancy between the prolongation of the clotting time of normal plasma by the patient's plasma, and the inability to demonstrate inhibition of prothrombin and accelerator activity by two-stage systems might be explained by the marked difference in plasma dilutions in the tests. Low accelerator values could be normalized repeatedly by normal human $BaSO_4$-adsorbed plasma but never by normal human 48 hour old serum. This not only indicated a deficiency of factor V, instead of factor VII, but also that an inhibitor was not operative against homologous factor V. Obviously, dilution was not a significant variable here. It is pertinent that Hörder has described a case of factor V deficiency in which a congenital familial inhibitor of this factor was demonstrated against purified factor V and factor V activity of normal human and $BaSO_4$-treated cow plasma (30). The patient never exhibited manifestations of hypersensitivity and consistently had a positive cephalin cholesterol flocculation, false positive complement fixation test for syphilis and a circulating anticoagulant. This triad, or just the circulating anticoagulant, not infrequently has been present in cases of systemic "collagen-vascular disease," particularly disseminated lupus erythematosus (31–33).

There have been few reports on the effect of methionine in blood coagulation, and many of these have been contradictory (34–38). The study most closely relevant to our work is that of Shin (39), who showed that the administration of L- and DL-methionine, orally or parenterally, to CCl_4- and $CHCl_3$-treated rabbits resulted in an increase in one-stage prothrombin activity [now generally considered to be a function of factors V and VII as well as of prothrombin (40)]. Vitamin B_{12}, sodium thiolactate and vitamin K had a similar effect. Vitamin K, administered concomitantly with methionine, was not reported, however.

The methionine intake in the normal diet is relatively large (approximately 4 Gm. per day), but doubling or tripling the intake in the form of chemically pure methionine effects a reduction of prothrombin and accelerator values (38). On the other hand, increasing the methionine to only 0.5 Gm. above the normal daily intake, in conjunction with vitamin K, results in an elevation of accelerator values. At first glance, it seems surprising to expect any effect from a 10 to 15 per cent increase of methionine intake above that present in a normal, or especially in a high protein or high methionine diet (Case 1) when digestion and absorption presumably are unimpaired. Methionine, for example, has been shown to have an insignificant nitrogen-sparing effect when the diet already contains large amounts of protein of good methionine content (41). A reasonable explanation for the effect of methionine supplementation is the assumption that the metabolism and end products of the administered methionine, unencumbered by proteins and other amino acids, are different from those of methionine present in natural foods. The relatively slow transformation from whole protein to amino acids and variation in absorption and mass action rates might well be factors which would determine a specific pathway different in the administered methionine from that present in a complex protein moiety.

The mode of action of the combined methionine and vitamin K and of vitamin K-S(II) effect on accelerator activity is unknown. The results obtained with vitamin K-S(II) do, however, substantiate our original hypothesis. It was postulated that since methionine and vitamin K acted synergistically, the mercapto-propyl radical substituted in the vitamin K molecule might be equally effective. Attention is directed to the structural similarity between this radical (SCH_2 CH_2 COOH) and a product of methionine catabolism. Although the mercapto-propyl radical is not one of the recognized cleavage products, recent studies have substantiated the formation of propionic acid in the catabolism of the methionine carbon chain in the rat (42). It might well be that certain true end products of methionine degradation such as

methyl mercaptan (43) might be equally effective or that methionine administered parenterally with vitamin K might be ineffective.

Although proof is lacking, our working hypothesis has been that the organic sulfur in the three-position of vitamin K-S(II) is important for its biological activity, and may account for that manifested by the methionine-vitamin K synergism also. It has been shown that in rats treated with ethionine, there is a marked depression of prothrombin, factor V, factor VII, antithrombin, to a lesser extent fibrinogen (44) and of antihemophilic globulin (45). Since ethionine, the ethyl analog of methionine, is a metabolic blocking agent which interferes with the synthesis of certain proteins, it seems reasonable to assume that this compound may act by competitively inhibiting methionine presumably essential for manufacture or activity of these clotting factors. With respect to accelerator activity at least, in both the experimental and clinical studies, the influence of methionine is apparent and would tend to substantiate the implications deduced from the ethionine experiments. Thus, to speculate one step further, it is possible that vitamin K-S(II) exerts its effect by contributing an essential mercapto-propyl radical as well as by its oxidizing propensity.

The ease with which sulfhydryl compounds may be added to naphthoquinones has been known for many years. Fieser and Turner (46) have prepared many thiol derivatives of 2-methyl-1, 4-naphthoquinone. The thioglycolic acid derivative was said to possess half the antihemorrhagic activity of vitamin K_1. In Case 1, prothrombin was refractory to all forms of therapy. In Case 2, both prothrombin and accelerator responded promptly to vitamin K-S(II). Although likely, it is not known whether these factors in this second case would have responded just as effectively to vitamin K_1. The etiology of the probable avitaminosis K in Case 2 is unknown. In any event, from both our experimental and clinical studies, it is apparent that vitamin K-S(II) does have some beneficial effect on prothrombin in vitamin K deficiency states.

SUMMARY

1. The accelerator response in blood coagulation to a new methyl naphthoquinone in cholecystnephrostomized dogs and in partially hepatectomized rats was striking. This compound, designated vitamin K-S(II), is S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercaptopropionic acid.

2. Administration of DL-methionine to normal dogs and rats effected a consistent but mild elevation of accelerator levels, but DL-methionine plus vitamin K always evoked an even greater response.

3. DL-methionine and vitamin K, administered separately to cholecystnephrostomized dogs, resulted in a moderate rise of accelerator levels; when given concomitantly, hypernormal levels were achieved.

4. Accelerator levels in cholecystnephrostomized dogs on protein-free and methionine-free diets became almost negligible. With the addition of DL-methionine to the diets, they promptly returned to normal on the former and gradually increased on the latter. A similar qualitative response was observed in normal dogs on the methionine-free diet.

5. In partially hepatectomized rats, the rate of recovery of accelerator activity was decidedly faster, and the time required to attain normal levels or above, two days less on methionine-supplemented diets than on the stock or menadione-supplemented diet.

6. A patient with a multifacetted hemorrhagic disorder, studied for six years, is presented. The etiology and pathogenesis remain obscure. The principal features included severe hemorrhagic manifestations, prolonged clotting times, hypoprothrombinemia, accelerator (factor V) deficiency, thrombocytopenia, hyperfibrinogenemia, a circulating anticoagulant, transient hypocalcemia, hemolytic anemia, elevated cephalin flocculation and false positive complement fixation tests. DL-methionine and vitamin K, administered concomitantly, repeatedly exerted a corrective effect on accelerator levels in this patient whereas fresh and banked blood and massive doses of vitamins K and K_1 had no effect. The synergistic effect was sustained so long as both methionine and vitamin K were given; neither one alone was capable of eliciting this response, nor once achieved, of maintaining it for long periods.

7. Vitamin K-S(II) similarly exerted a corrective effect on accelerator activity in this same patient.

8. The third recorded case of hypoprothrombinemia and "hypoproconvertinemia" in pregnancy is presented. This patient, in addition, had a factor V deficiency. All of these deficiencies were corrected by vitamin K-S(II).

9. Hemorrhagic manifestations disappeared completely when the patients were treated with the above compounds, and the correlation of this therapy with normal accelerator levels and freedom from bleeding in Case 1 was impressive.

10. In contrast to the effect of vitamin K-S(II) on accelerator activity, that on prothrombin was negligible except in vitamin K deficiency states.

11. Both methionine and vitamin K, and vitamin K-S(II) were well tolerated and no toxic manifestations were encountered.

CONCLUSIONS

Analysis of the experimental and clinical data indicates that accelerator activity in blood coagulation is influenced by the concomitant administration of methionine and vitamin K and by vitamin K-S(II). These compounds would appear to be useful, at times even specific, therapeutic measures in the treatment and management of accelerator deficiencies. Determination of the extent of applicability of these measures must await further clinical trial.

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THE ULTRAFILTRABLE CALCIUM OF HUMAN SERUM. II. VARIATIONS IN DISEASE STATES AND UNDER EXPERIMENTAL CONDITIONS [1]

By A. RAYMOND TEREPKA, T. Y. TORIBARA, AND PRISCILLA A. DEWEY

(From the Department of Medicine and the Division of Pharmacology of the Department of Radiation Biology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

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The total serum calcium is composed of two major fractions: the non-diffusible or protein bound calcium, and the diffusible or ultrafiltrable calcium. The latter, containing predominantly ionic calcium, theoretically traverses capillary membranes *in vivo* and participates in metabolic processes at the cellular level.

In a previous publication, a new simple method for the ultrafiltration of human serum was described (1). It was found that in normal adults, between 60 and 70 per cent of the calcium present in serum was ultrafiltrable. These findings were compared with the variable values reported by other authors for the normal percentage of ultrafiltrable calcium. It was pointed out that virtually all previous workers had failed to control both the pH and the temperature during their ultrafiltration procedures. Our data indicated that these variables had a considerable effect on the ultrafiltrability of serum calcium, thus offering an explanation for the lack of agreement, particularly among the early workers (2), in the study of normal and abnormal sera. Only the recent data of Hopkins, Connor, and Howard (3) show reasonable consistency for specific disease states. They used the Lavietes mercury-pressure ultrafiltration apparatus, controlled pH, and carried out the ultrafiltration procedure at room temperature.

Utilizing the controlled technique previously described (1) we have reinvestigated the distribution of calcium in the serum of a group of patients with hypercalcemia and hypocalcemia. These results are given in the first portion of this communication. Although differing in the absolute quantity, and therefore the per cent of ultrafiltrable calcium, our findings are in agreement with those reported by Hopkins and her associates (3).[2] However, certain interesting observations in renal disease prompted us to investigate more closely the hypocalcemia associated with this condition.

The ultrafiltration method we have utilized requires relatively small quantities of serum (3 ml.). Thus, frequent determinations of ultrafiltrable calcium are possible during experimental procedures. To supplement the observations in disease states, a detailed investigation was made of acute hypercalcemia in human subjects induced by calcium infusion or parathyroid extract administration. In addition, hyperphosphatemia and hypercitremia, both considered to have specific effects on total or ultrafiltrable calcium, were induced experimentally and observations made on the distribution of calcium in serum. The results of these experiments are presented in the second portion of this paper.

METHODS

Patients on the general medical wards of the Strong Memorial Hospital with abnormal total serum calcium values were selected for the study of the distribution of serum calcium in disease states. A definite diagnosis was established clinically in all cases. Blood was collected by venipuncture in the non-fasting state and serum ultrafiltered the same day. Another patient, who was being studied on the metabolism ward of the Department of Medicine, served as the subject for the detailed investigation of the effect of experimentally induced conditions on the ultrafiltrability of serum calcium. He was a 75 year old white man with chronic lymphatic leukemia and an annular carcinoma of the rectosigmoid. In spite of the two malignant processes, he appeared well nourished (body weight, 63 Kg.) and was generally asymptomatic but did have slight hypoproteinemia. No evidence of bone involvement with cancer was apparent clinically, by X-ray or balance study.

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

[2] The higher values for ultrafiltrable calcium in the studies of Hopkins, Connor, and Howard (3) probably resulted because these authors carried out their ultrafiltration procedures at room temperature instead of 37° C.

TABLE I

Ultrafiltration results and other pertinent data in patients with hypercalcemia and hypocalcemia

Subject	Age	Diagnosis	Date	Serum calcium*			Serum P*	Serum protein†		
				Total	Ultrafil.	% U.F.		Total	Alb.	Glob.
M. M.	60	Vit. D intoxication	1/24	11.57	8.34	72.2	3.0	6.6	4.4	2.2
C. N.	76	Vit. D intoxication	6/3	12.50	8.97	71.7	5.1	7.1	4.9	2.2
J. V.	34	Boeck's sarcoidosis	2/2	11.19	7.84	70.1	3.2	8.7	4.0	4.7
			2/7	11.30	8.08	71.5	3.3			
			3/2	11.31	8.03	71.0	2.8	8.6	4.0	4.6
E. H.	63	Breast carcinoma	7/9	12.50	8.80	70.3		7.0	5.2	1.8
E. T.	53	Breast carcinoma	2/7	16.50	10.07	65.0	6.6	7.2	4.0	3.2
			2/13	13.28	9.05	68.1	4.1			
			2/17	12.45	8.03	64.5	2.2	7.5	4.7	2.8
			2/24	11.40	7.67	67.2	2.5	7.0	4.3	2.7
			3/8	22.90	12.40	54.2	5.0			
F. D.	20	Secondary hyperparathyroidism	6/17	10.18	6.39	62.8	11.5	5.1	3.5	1.6
A. S.	43	Hypoparathyroidism (post op.)	5/31	6.32	4.17	65.9	7.5	6.9	4.1	2.8
M. P.	59	Hypoparathyroidism (idiopathic)	6/27	4.48	3.40	75.8		8.3	4.7	3.6
			6/28	5.17	3.77	72.9	7.14			
			6/29	5.38	3.63	67.4		7.3	4.2	3.1
			7/3	5.68	3.70	65.2	6.12	7.6	4.1	3.5
			7/6	5.58	3.70	66.3	6.37			
			7/7	6.18			4.85			
			7/9	6.18			4.44			
			7/17‡	6.46	3.96	61.3	2.68	6.0	3.1	2.9
				7.37	4.58	62.2		6.3	3.0	3.3
				7.50				6.7	3.1	3.6
				7.64	4.78	62.6		6.4	3.1	3.3
C. W.	24	Steatorrhea	4/8	5.83	5.10	87.4	1.9	4.6	2.5	2.1
J. A.	78	Malnutrition Liver disease	5/23	7.73	6.48	83.8	3.2	5.8	2.0	3.8

* Values in mg. per cent.
† Values in Gm. per cent.
‡ Blood samples obtained at 9:00 a.m., 10:30 a.m., 11:45 a.m. and 2:00 p.m.

The procedure for the ultrafiltration of human serum was described in detail in a previous paper (1). All determinations were done at 36° C. after equilibration of the apparatus with 5 per cent carbon dioxide and 95 per cent oxygen. Calcium was determined in both whole serum and the ultrafiltrates by a flame photometric technique (4). The normal range for total serum calcium and ultrafiltrable calcium by this method is 9.26 to 10.30 mg. per cent and 6.17 to 7.16 mg. per cent, respectively. The "per cent of ultrafiltrable calcium" was obtained by simply dividing the concentration of calcium in the ultrafiltrate by that in the whole serum (no protein correction) and multiplying by 100 per cent.

Inorganic phosphorus determinations on the serum of patients with hypocalcemia and hypercalcemia were done by the Fiske and Subbarow method (5) while the method of Chen, Toribara, and Warner (6) was used for the determination of total serum phosphorus and serum inorganic phosphorus in the patient studied on the metabolism ward and in patient M.P. (Table I) with idiopathic hypoparathyroidism. Total carbon dioxide content of serum and blood urea nitrogen were determined by the Van Slyke and Neill (7) and the Van Slyke and Cullen (8) methods, respectively. Fractionation of the serum proteins was carried out by a detergent technique (9) and the serum citrate analyses were done by the method of Ettinger, Goldbaum, and Smith (10).[3] The latter two methods give the following values for normal sera: total protein, 6.5 to 8.0 Gm. per cent; albumin, 4.5 to 5.5 Gm. per cent; total serum citrate, 3 to 5 mg. per cent.

RESULTS

I. The serum ultrafiltrable calcium in disease states

A. Hypercalcemia

The ultrafiltrable calcium in a variety of diseases associated with abnormalities in serum calcium are recorded in Tables I and II along with other pertinent data. Normal human subjects have ultrafiltrable calciums ranging between 61.7 and 71.4 per cent of the total serum calcium (1) so that the concentration of ultrafiltrable calcium

[3] The authors are indebted to Dr. Ralph Jacox for the protein determinations and Betty Jane Mulryan for the citrate analyses.

ranges between 6.2 and 7.2 mg. per cent. It can be seen in Table I that in the patients with hypervitaminosis D, Boeck's sarcoidosis, and metastatic breast carcinoma with hypercalcemia, the concentration of ultrafiltrable calcium was distinctly elevated, while the percentage of the total serum calcium which was ultrafiltrable was in, or only slightly above, the normal range. These patients tended to have mild degrees of hypoalbuminemia, while the total serum protein concentration was variable.

One of the patients with carcinoma of the breast (E.T.) was studied during a cortisone induced remission of hypercalcemia and after the hypercalcemia had rapidly recurred (3/8 sample) during an attempt at pallative estrogen treatment. The fall in total serum calcium during cortisone administration was accompanied by a proportionate decrease in both ultrafiltrable and non-ultrafiltrable calcium so that the per cent of ultrafiltrable calcium remained essentially constant and within the normal range. However, the extreme hypercalcemia induced by the estrogen administration was associated with an abnormally low percentage of ultrafiltrable calcium. A similar decrease in the ultrafiltrability of calcium in extreme hypercalcemia in man has been noted by others (3) and has been attributed to the formation of a "colloidal calcium-phosphate-protein complex" (11).

Patient F.D., with secondary hyperparathyroidism, had a congenital urinary tract anomaly and long standing renal disease. Typical destructive lesions of osteitis fibrosa cystica were seen in X-rays of the hands and skull. Metastatic calcifications of arteries and soft tissues were prominent and the vertebrae showed the interesting combination of simultaneous destructive and sclerotic changes (12). His total serum calcium concentration and the concentration and percentage of ultrafiltrable calcium were normal. The significance of these observations will be left for the discussion after the results of the calcium infusion and parathyroid extract administration are presented.

B. Hypocalcemia

The hypocalcemic conditions studied have been arbitrarily divided into those without (Table I) and with (Table II) significant renal disease. Patient A.S. (Table I) was studied seven days after a sub-total thyroidectomy for toxic nodular goiter. Two days post-operatively he was found to have a positive Trousseau's sign and his serum calcium was 6.0 mg. per cent. Treatment with calcium, vitamin D and parathyroid extract was started and at the time of study he was receiving 12 Gm. of calcium gluconate and 75,000 units of vitamin D orally each day. Signs of latent tetany were still present. Both his total serum calcium and ultrafiltrable calcium were low, and they had decreased proportionately so that the per cent of ultrafiltrable calcium was similar to that found in the normal individuals. Note that his serum proteins were essentially normal.

Patient M.P. was studied before and during therapy for idiopathic hypoparathyroidism. Treat-

TABLE II

Ultrafiltration results and other pertinent data in patients with chronic renal disease

Subject	Age	Diagnosis	Serum calcium*			Serum P*	BUN*	Serum CO_2†	Serum protein‡		
			Total	Ultrafil.	% U.F.				Total	Alb.	Glob.
G. L.	48	Glomerulonephritis	6.66	5.58	83.6	12.0	83	21	5.8	3.1	2.7
C. S.	21	Chronic pyelonephritis	7.84	6.46	82.4	14.5	148	16	4.8	3.2	1.6
F. G.	75	Chronic pyelonephritis	7.98	6.19	77.4	6.2	82	16	6.2	3.8	2.4
D. F.	36	Kimmelstiel-Wilson Syn.	8.03	5.99	74.6	7.2	107	19	7.0	3.4	3.6
A. V.	51	Kimmelstiel-Wilson Syn.	8.10	6.46	79.7	3.9	48	20	5.1	2.5	2.6
N. O.	58	Glomerulonephritis	8.12	7.40	91.2	13.1	210	13	7.5	5.2	2.3
E. M.	77	Gouty nephritis	8.81	6.53	74.1	6.5	105	15	7.8	5.4	2.4
T. G.	31	Chronic pyelonephritis	9.03	6.63	73.5		63	19	7.6	4.5	3.1
L. W.	56	Chronic pyelonephritis	9.69	7.70	79.4	5.4	65	19	5.7	3.1	2.6
R. W.	65	Glomerulonephritis	5.58	3.81	68.3	5.0	122	26	5.7	3.4	2.3
C. E.	88	Nephrosclerosis	7.45	5.04	67.7	6.7	105	26	5.3	3.0	2.3

* Values in mg. per cent.
† Values in mM per L.
‡ Values in Gm. per cent.

ment with 3.75 mg. of A.T. 10 and 6 Gm. of calcium gluconate per day, orally, were started on 6/28. It can be seen that the initial treatment with calcium and A.T. 10 was associated with a gradual rise in total serum calcium and little rise in ultrafiltrable calcium so that the per cent of ultrafiltrable calcium fell significantly. Vitamin D, 150,000 units per day, orally, was started on 7/1 and the A.T. 10 discontinued. This patient continued to have intermittent severe convulsive episodes until 7/4. It should be noted that on 7/17, after more than two weeks of therapy with calcium and vitamin D, the ultrafiltrable calcium had increased only about 0.5 mg. per cent while the total calcium had increased 2 mg. per cent. However, when parathyroid hormone was administered during an Ellsworth-Howard test (13) (done on 7/17), both the total calcium and ultrafiltrable calcium increased approximately 1 mg. per cent in a matter of five hours and the per cent of ultrafiltrable calcium remained constant.

Patient C.W. was admitted to the hospital for an acute exacerbation of steatorrhea. She had a very low total serum calcium on admission but a near normal concentration of ultrafiltrable calcium. Almost 90 per cent of the calcium in her serum was ultrafiltrable. At no time did she manifest signs or symptoms of latent tetany. Of significance is the fact that both the total protein and albumin concentrations in her serum were markedly reduced. Similarly, in patient J.A., with very low serum proteins due to liver disease, the per cent of ultrafiltrable calcium was 84 and the concentration of calcium in the serum ultrafiltrate was actually normal, although the total serum calcium was approximately 2 mg. per cent below normal.

In Table II we have grouped together the patients with hypocalcemia secondary to chronic renal disease. It is immediately apparent that the percentage of ultrafiltrable calcium in all except the last two patients (R.W. and C.E.) was abnormally high, regardless of the level of total calcium. Consequently, the concentration of calcium in the serum ultrafiltrates of these patients was within or near the normal range and, in several instances, was greater than normal. None of them, including R.W. and C.E., exhibited signs or symptoms of latent tetany.

Other pertinent blood chemistry determinations in these patients are also listed in the table. The blood urea nitrogen and serum inorganic phosphorus were elevated in all subjects but to varying degrees. Notice the inconsistent correlation between the levels of serum inorganic phosphorus and total serum protein and the degree of hypocalcemia. Although, in general, the serum proteins tended to be low, especially the albumin fraction, in three patients with a high percentage of ultrafiltrable calcium, N.O., E.M. and T.G., these were normal. Of possible significance is the fact that the carbon dioxide content of the serum was normal in R.W. and C.E., who showed no increase in the per cent of ultrafiltrable calcium, as contrasted with the other nine patients where it was uniformly decreased. In both these patients, alkali therapy had corrected the metabolic acidosis which had been present at the time of admission to the hospital. The implications of these findings will be discussed below.

II. The serum ultrafiltrable calcium under experimental conditions

A. Calcium infusion and parathyroid extract administration

Our ultrafiltration results in disease states associated with hypercalcemia (Table I) and those of Hopkins, Connor, and Howard (3) indicate that the additional calcium is distributed proportionately between both ultrafiltrable and protein-bound calcium. Consequently, the percentage of the total serum calcium that is ultrafiltrable is generally within the range found for normal subjects. Since these clinical conditions represent, for the most part, chronic alterations in calcium and perhaps protein metabolism, it seemed of interest to compare them with a hypercalcemia acutely induced by the administration of "ionic calcium" or parathyroid extract. Figures 1 and 2 show the results of these experiments.

Nine Gm. of calcium gluconate-heptonate in 900 ml. of 5 per cent dextrose in water (890 mg. of calcium) was infused intravenously in a human subject over a three hour period and blood samples obtained at the times indicated in Figure 1. The total serum calcium was increased from 9.0 to 12.6 mg. per cent during the infusion, began to fall immediately after the infusion was stopped, and the next morning was still well above the pre-

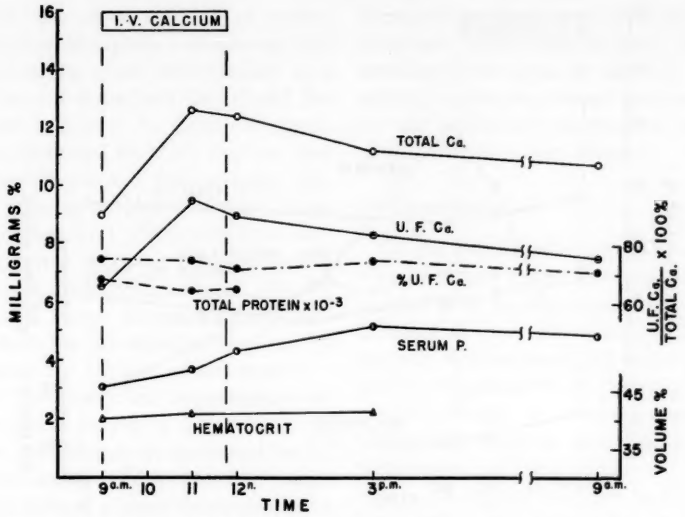
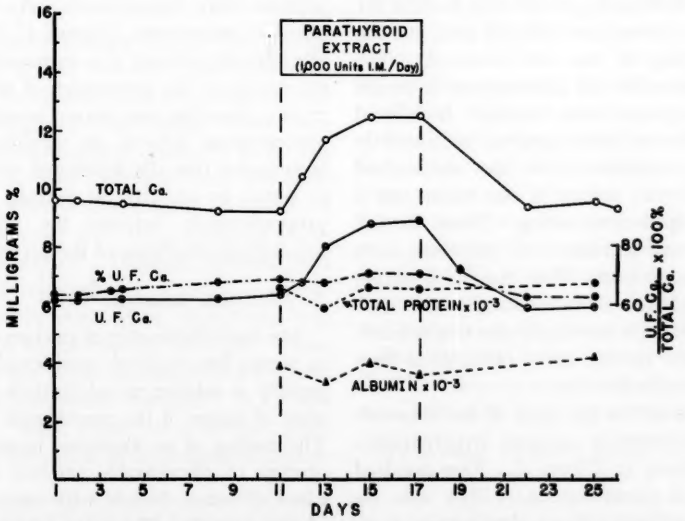


FIG. 1. THE EFFECT OF INTRAVENOUS CALCIUM GLUCONATE (14 MG. CALCIUM PER KG. B.W.) ON ULTRAFILTRABLE CALCIUM

The right ordinate refers to the hematocrit and the per cent of ultrafiltrable calcium (% U.F.Ca) and the left ordinate to all others.

injection level. Although the quantity of ultrafiltrable calcium also increased markedly, it should be noted that it remained a constant percentage of the total serum calcium. Consequently, the quantity of calcium bound by the serum proteins (*i.e.*, non-ultrafiltrable) must also have increased proportionately although there was, if anything, a slight fall in the total protein concentration (no


PARATHYROID EXTRACT (1000 Units I.M./Day)
TOTAL Ca.
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U. F. Ca.
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ALBUMIN x 10-3
MILLIGRAMS %
U.F.Ca. / TOTAL Ca. x 100%
1 3 5 7 9 11 13 15 17 19 21 23 25
DAYS

FIG. 2. THE EFFECT OF PARATHYROID EXTRACT ON ULTRAFILTRABLE CALCIUM

The right ordinate refers to the per cent of ultrafiltrable calcium (% U.F.Ca) and the left ordinate to all others.

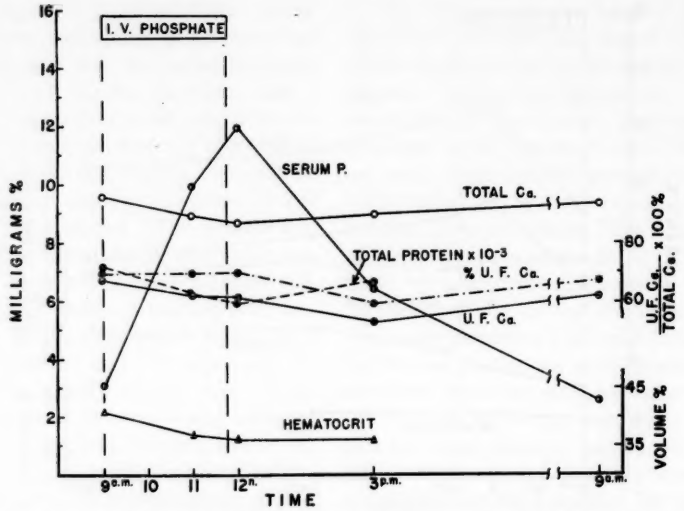

FIG. 3. THE EFFECT OF INTRAVENOUS SODIUM PHOSPHATE (34 MG. PHOSPHORUS PER KG. B.W.) ON ULTRAFILTRABLE CALCIUM

The right ordinate refers to the hematocrit and the per cent of ultrafiltrable calcium (% U.F.Ca) and the left ordinate to all others.

significant change in serum albumin: globulin [A/G] ratio occurred).

A gradual and persistent rise in serum inorganic phosphorus also was noted. This increased 2.1 mg. per cent, from a pre-injection level of 3.1 mg. per cent to 5.2 mg. per cent at 3 p.m., and the following morning it was still elevated. The mechanism responsible for this increase in serum inorganic phosphorus when calcium is infused intravenously has not been explained satisfactorily (14). In our experiment, we also determined the total phosphorus content of the 9 a.m. and 3 p.m. serum samples after ashing. These showed a 2.8 mg. per cent increase over this time, from 11.6 to 14.4 mg. per cent. Thus, the rise in serum inorganic phosphorus characteristically seen during calcium infusion is not simply due to a redistribution among the various serum phosphorus fractions but is a specific increase.

The data obtained in the study of the hypercalcemia of experimentally induced hyperparathyroidism are shown in Figure 2. Five hundred units (5 ml.) of parathormone (Lilly) were injected intramuscularly twice a day for six days. The subject was maintained on a constant dietary intake of calcium, phosphorus and nitrogen throughout the experiment. During the period of parathyroid extract administration, the total serum calcium increased to 12.5 mg. per cent and then promptly fell to control levels when the injections were discontinued. As in the calcium infusion experiment (Figure 1) the quantity of ultrafiltrable calcium also increased but there was no change in the percentage of ultrafiltrable calcium. Since the total serum protein and albumin concentration showed no significant changes, it is apparent that the additional calcium mobilized to serum by parathyroid extract was distributed proportionately between the ultrafiltrable and non-filtrable fractions of the serum calcium.

B. Phosphate infusion

The interrelationship of calcium and phosphorus in serum has received considerable attention, especially in relation to calcification and the mechanism of action of the parathyroid hormone (15). The finding of an abnormal increase in the percentage of ultrafiltrable calcium in most of our cases of renal disease with nitrogen and phosphorus retention (Table II), prompted us to study the effect of acute hyperphosphatemia *per se* on the distribution of calcium in serum.

Nine hundred ml. of a solution of monosodium and disodium phosphate [4] containing 2.17 Gm. of phosphorus was given intravenously to a human subject over a three hour period and the results are shown in Figure 3. Serum inorganic phosphorus was increased to 11.95 mg. per cent at the end of the infusion and then promptly fell. The following morning it was somewhat lower than the pre-injection level. This should be contrasted with the serum inorganic phosphorus values in the calcium infusion experiment (Figure 1). In the latter, serum inorganic phosphorus was still well above the pre-injection level on the morning following the calcium administration.

A fall in total serum calcium concentration occurred coincident with the rise in serum inorganic phosphorus, but this fall may be accounted for on the basis of hemodilution. Blood hematocrits done during the study showed a rapid decrease from a control level of 41 to 36 per cent at the end of the infusion and the total protein concentration fell from 7.2 to 6.0 Gm. per cent over the same period (no change in serum A/G ratio occurred). The decrease in total serum calcium during the infusion was accompanied by a proportionate fall in ultrafiltrable calcium so that the per cent of the serum calcium that was ultrafiltrable remained constant throughout the period of marked hyperphosphatemia.

Our data indicate a sharp fall in ultrafiltrable calcium in the 3 p.m. sample. Since the total serum calcium was almost back to normal by this time, the percentage of ultrafiltrable calcium also shows a significant decrease (from 70 to 59 per cent). The following morning both the total calcium and the ultrafiltrable calcium had returned to pre-injection levels and the per cent of ultrafiltrable calcium was 67. Hopkins, Howard, and Eisenberg (16) reported a similar fall in ultrafiltrable calcium *in vitro* when phosphorus was added to serum in increasing amounts. These authors related this to the formation of a colloidal "calcium-phosphate-protein complex" (11) and suggested that "there might be a delayed equilibrium in the formation of a calcium-phosphate 'complex,' as there is in precipitation of calcium phosphate from supersaturated solutions." Interestingly, the apparent decrease in ultrafiltrable calcium in our experiment occurred sometime after the maximum elevation in serum inorganic phosphorus had been attained.

[4] The solution contained 2.14 Gm. of $NaH_2PO_4 \cdot H_2O$ and 8.83 Gm. of Na_2HPO_4 per L. of 5 per cent dextrose in water. The sodium concentration, determined by flame photometry, was 136 mEq. per L. and the pH was 7.36 when measured on a Beckman model G pH meter.

C. Citrate infusion

Of the ultrafiltrable anionic complexers of calcium in serum, citrate has received most attention because of its possible relationship to calcium metabolism in bone and kidney. It has been repeatedly demonstrated that a rise or fall in serum calcium concentration is generally associated with parallel changes in serum citrate levels (17). Chang and Freeman also found (18) that the intravenous administration of sodium citrate (given to dogs at the rate of 0.67 mg. per Kg. body weight per minute for two hours) caused an increase in total serum calcium concentration. On the other hand, hypercitremia from rapid transfusion of citrated whole blood has been associated with signs and symptoms of tetany, presumably on the basis of acute depression of ionized calcium (19).

We investigated the effects of an intravenous infusion of approximately 0.5 mg. of citrate ion per Kg. body weight per minute on total and ultrafiltrable calcium in a human subject.[5] Seven hundred ml. of a 5 per cent dextrose in water solution containing 9.10 Gm. of sodium citrate (5.85 Gm. of citrate ion and 134 mEq. per L. of sodium) was infused intravenously over a three hour period and blood samples were obtained before, during and after the infusion (Figure 4). Serum citrate was increased about fivefold to 15.5 mg. per cent. The rapidity of citrate metabolism *in vivo* (21) is demonstrated by the fact that three hours later the serum citrate concentration had returned to the pre-injection level.

Both the total serum protein concentrations and the blood hematocrits fell rapidly during the infusion, indicating acute hemodilution. Total serum

[5] In a recent review of citrate intoxication (20), it was stated that citrate injections at the rate of 1.0 mg. per Kg. body weight per minute in man may lead to toxic manifestations, although the rate usually attained in infants who developed tetany during exchange transfusion has been between 5 and 10 mg. of citrate per Kg. body weight per minute.

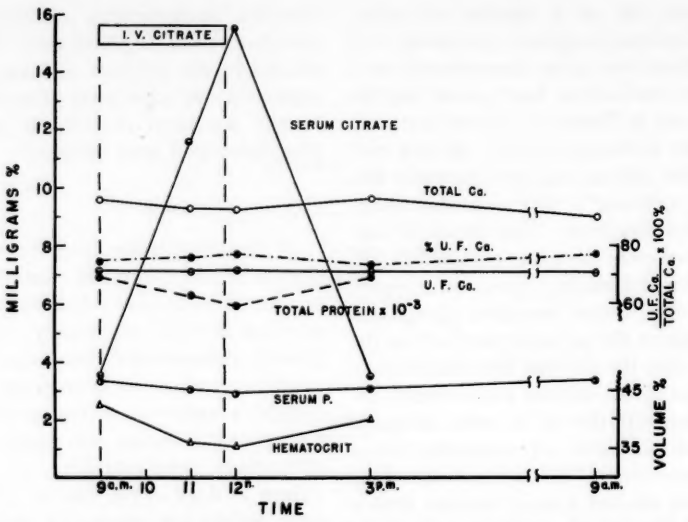

FIG. 4. THE EFFECT OF INTRAVENOUS SODIUM CITRATE (0.5 MG. CITRATE ION PER KG. B.W. PER MINUTE) ON ULTRAFILTRABLE CALCIUM

The right ordinate refers to the hematocrit and the per cent of ultrafiltrable calcium (% U.F.Ca) and the left ordinate to all others.

calcium decreased, but not as much as might be expected on the basis of the hemodilution (*cf.*, the fall in total serum calcium in the phosphate infusion experiment shown in Figure 3). Since Chang and Freeman (18) used relatively small volumes of fluid to induce hypercitremia in their dogs, the hemodilution in our experiment could have masked an actual increase in serum calcium concentration. If the recently suggested hypothesis (22, 23) that citrate has a primary role in the mobilization of calcium from bone to the extracellular fluid is correct, high blood citrate levels might be expected to increase serum calcium by causing a more rapid release of calcium from bone.

By using the dissociation constant of calcium citrate, the quantities of citrate bound calcium in serum may be calculated.[6] At the initial serum citrate concentration of 3.5 mg. per cent approximately 0.5 mg. per cent of calcium is complexed as diffusible calcium citrate. An increase in serum citrate concentration to 15.5 mg. per cent (12 o'clock noon level) will result in a minimum of 2 mg. per cent of citrate complexed calcium. The increase in calcium bound to citrate must be supplied from the pool consisting of protein bound calcium, ionic calcium, and complexed but diffusible calcium. An appreciable decrease in protein bound calcium would result in an increase in ultrafiltrable calcium. As can be seen in Figure 4, only a minimal increase in the percentage of ultrafiltrable calcium occurred even at the height of the hypercitremia. Thus, most of the additional calcium complexed by citrate must have come from the diffusible forms of calcium. "Ionic" calcium, because of its relative abundance in serum (25, 26), would undoubtedly be most affected.

[6] This calculation can be made by utilizing the equation:

$$[\text{Ca Cit}^-] = \frac{K \cdot T\,[\text{Ca}^{++}]}{1 + K\,[\text{Ca}^{++}]}$$

where K equals stability constant of the calcium citrate complex and T equals total citrate. This equation is obtained by solving simultaneously the two equations:

$$K = \frac{[\text{Ca Cit}^-]}{[\text{Ca}^{++}][\text{Cit}^-]} \quad \text{and} \quad T = [\text{Ca Cit}^-] + [\text{Cit}^{\equiv}]$$

The values 1.25 mM per L. for $[Ca^{++}]$ and 1.66×10^9 for K, of Hastings, McLean, Eichelberger, Hall, and Da Costa (24), were used in our calculations.

DISCUSSION

Ultrafiltration results may be expressed in two ways: 1) as the actual concentration of calcium in mg. per cent in the ultrafiltrates of whole serum, or 2) as the percentage of the total calcium

in the serum that is ultrafiltrable. Most previous authors, including ourselves, have emphasized either one or the other (rarely both) in their writings on ultrafiltrable calcium in normal and disease states. We believe that both methods of expressing ultrafiltration results are equally meaningful but, as discussed below, variations from normal values in the percentage or concentration of ultrafiltrable calcium are dependent upon different physiological mechanisms.

McLean and Hastings (27) postulated in 1935 that the distribution of calcium in serum, between ultrafiltrable and non-ultrafiltrable forms, is governed by a purely physical chemical process: the mass action relationship $Ca^{++} + Prot \rightleftharpoons CaProteinate$. Our findings, and those of others (3, 14), in hypercalcemia, whether secondary to a disease process (Table I) or experimentally induced by the administration of calcium (Figure 1) or parathyroid extract (Figure 2), support this hypothesis. Calcium added to serum *in vivo* under any of these circumstances led not only to an increase in ultrafiltrate calcium but to a proportionate increase in protein bound calcium so that the per cent of calcium which was ultrafiltrable was not altered significantly. This relationship has also been clearly demonstrated, *in vitro,* by adding increasing amounts of calcium to serum before ultrafiltration (16).

It is generally accepted that the parathyroid glands are intimately concerned with the maintenance of a normal serum calcium concentration. Although the precise physiological mechanisms involved are still incompletely understood, it is now believed that the parathyroids are concerned with the equilibrium between calcium in bone and extracellular fluid (28–31). Once in the serum, however, the calcium released from bone under parathyroid influence also obeys the law of mass action in its reaction with serum proteins. Thus, when the bone: extracellular fluid equilibrium was disturbed by the administration of parathyroid extract (Figure 2, and M.P., Table I) both the ultrafiltrable calcium and the total calcium increased proportionately so that the per cent of ultrafiltrable calcium was not altered, while in hypoparathyroidism (A.S. and M.P., Table I of our data, and Table III of Hopkins, Connor, and Howard [3]), there was a proportionate decrease in both these calcium fractions. On the other hand, hypoproteinemia (C.N. and J.A., Table I), with presumably normal parathyroid function, was associated with a low total serum calcium, a normal or near normal concentration of ultrafiltrable calcium and a high percentage of ultrafiltrable calcium. These considerations indicate that the *concentration* of calcium in ultrafiltrates of serum is determined by the bone: extracellular fluid calcium equilibrium, while the total serum calcium, and consequently the *per cent* of ultrafiltrable calcium, is secondarily determined by mass law relationships in serum or plasma. It follows that the total calcium in serum only reflects the level necessary to maintain the concentration of ultrafiltrable or ionized calcium determined by the parathyroid controlled bone: extracellular fluid equilibrium on the one hand, and serum mass law relationship on the other.

Both our data (Table I) and that of Hopkins, Connor, and Howard (3) showed that the hypercalcemia of malignant disease, Boeck's sarcoidosis, and vitamin D intoxication was associated with elevated concentrations of ultrafiltrable calcium, while the percentage of the total serum calcium that was ultrafiltrable was generally within the range found for normal subjects when serum proteins were essentially normal. Furthermore, when the hypercalcemia in one patient (E.T., Table I) was partially corrected with cortisone, the ultrafiltrable and total calcium fell proportionately and there was no change in the percentage of ultrafiltrable calcium. Similar findings in cortisone or adrenocorticotropic hormone therapy of hypercalcemia were reported by Hopkins and her associates (3). We interpret these observations to mean that the calcium disturbance in these disease states is also due to a primary alteration in the bone: extracellular fluid calcium equilibrium (which can be corrected with cortisone) with secondary changes in the total serum calcium. In this connection, it is of interest that several authors have recently reported (32, 33) cases of hypercalcemia in malignant disease without evidence of tumor invasion of bone (and with normal parathyroid glands at autopsy) although the hypercalcemia is usually ascribed to bone metastasis with rapid destruction of bone and release of calcium into the circulation.

From the above discussions, one may postulate that an alteration in the *concentration* of ultra-

filtrable calcium means some disturbance (albeit non-specific) in the calcium equilibrium at the skeletal level, while an abnormal *percentage* of ultrafiltrable calcium indicates an alteration in calcium-protein relationships in serum.

It is well known that hypocalcemia is often associated with chronic renal disease but that tetany is infrequently seen. The former has been attributed to hypoproteinemia and hyperphosphatemia, and the latter to acidosis, all of which frequently accompany these disease states. We could not correlate the degree of hypocalcemia in our patients with renal disease with the level of serum inorganic phosphorus and, although serum proteins tended to be low in the patients studied, this did not necessarily agree with the total calcium level. Our data, however, demonstrated (Table II) that in all but two of the patients studied, the percentage of the total serum calcium that was ultrafiltrable was abnormally high. Consequently, the quantity of ultrafiltrable calcium was essentially normal or even greater than normal (thus accounting for the clinical observations that tetany occurs infrequently in renal disease) while the amount of calcium bound to protein was decreased irrespective of the level of total calcium. The decrease in protein bound calcium was responsible for the hypocalcemia in most of these cases when the latter was present.

A decrease in protein bound calcium may occur because of: 1) a fall in concentration of serum protein, 2) a specific alteration in the binding ability of the proteins, or 3) a decrease in the ionized calcium level. In our patients, the high percentage of ultrafiltrable calcium did not correlate well with the level of total proteins or albumin in the serum. One or both of the other factors must also have been involved in decreasing protein bound calcium, but from the data at hand, we could not define the exact mechanisms.

It has been repeatedly demonstrated that pH will affect the binding ability of serum proteins (34, 35). However, as pointed out in our previous publication (1), the pH change accompanying the metabolic acidosis of renal disease should have only a minor effect on the ultrafiltrability of calcium in serum. Yet it seems significant that the two patients with low concentrations and normal percentages of ultrafiltrable calciums (R.W. and C.E.) were those whose "metabolic acidosis" had been treated and corrected, at least as judged by the serum carbon dioxide content. Some other change in chronic renal disease aside from the serum pH *per se,* but associated with the clinical state called "metabolic acidosis," could conceivably alter the ability of the serum proteins to bind calcium and contribute to the maintenance of a normal concentration of ultrafiltrable calcium.

The possibility that patients with renal disease and azotemia and hyperphosphatemia actually have low ionized calciums because of abnormal amounts of citrate in the blood was not substantiated (36). Total organic acids, however, were not measured, and we do not know the quantity of "unmeasured anions" (37) in our patients. It is possible that other abnormal calcium complexers are present which depress the ionic calcium and, therefore, protein bound calcium specifically. A detailed investigation of calcium-protein interrelationships in normal and disease states is in progress and will be reported in a separate communication.

SUMMARY

1. The ultrafiltrability of serum calcium in human subjects was determined: a) in disease states associated with hypercalcemia and hypocalcemia, and b) before, during and after the parenteral administration of calcium, citrate, phosphate and parathyroid extract.

2. An increase in serum calcium, whether due to a disease process or secondary to calcium or parathyroid extract administration, caused a proportionate increase in both ultrafiltrable and protein bound calcium so that no significant change occurred in the percentage of ultrafiltrable calcium.

3. Hypocalcemia was accompanied by normal or, more frequently, a high percentage of ultrafiltrable calcium. The latter was generally associated with hypoproteinemia, and the former with normal plasma proteins. This relationship, however, did not always hold in the hypocalcemia of renal disease.

4. In renal disease, a high percentage of ultrafiltrable calcium was almost invariable, regardless of the level of serum proteins so that the actual quantity of ultrafiltrable calcium approached normal. A specific alteration in the amount of calcium bound to serum proteins appeared to be present.

5. Hyperphosphatemia and hypercitremia had specific, but transient, effects on ultrafiltrable calcium.

6. By the application of physical chemical principles, abnormalities in the *concentration* of ultrafiltrable calcium in serum have been ascribed to disturbances in the bone: extracellular fluid calcium equilibrium, while abnormalities in the *percentage* of ultrafiltrable calcium (ratio of ultrafiltrable to total serum calcium) have been related to alterations in calcium-protein interrelationships in serum. In renal disease with disturbances in calcium metabolism, no simple mechanism correlated with the ultrafiltrable calcium values obtained.

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THE EFFECT OF ACETYLCHOLINE ON THE HUMAN PULMONARY CIRCULATION UNDER NORMAL AND HYPOXIC CONDITIONS [1]

By H. W. FRITTS, Jr.,[2] P. HARRIS,[3] R. H. CLAUSS, J. E. ODELL,[4] and A. COURNAND

(From the Departments of Medicine and Surgery, Columbia University College of Physicians and Surgeons, and the Cardio-Pulmonary Laboratory of the First Medical and Chest Services, Columbia University Division, Bellevue Hospital, New York, N. Y.)

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It is generally accepted that the tone of the peripheral arterioles plays an important part in regulating the systemic blood pressure in man. Whether the small vessels of the lungs exercise similar control over the pressure in the pulmonary artery is not so certain. This uncertainty has stemmed largely from the fact that the human pulmonary vessels have exhibited an erratic response to many vasoactive drugs (1–15). As a consequence, most physiologists have concluded either that the pulmonary vessels are incapable of intrinsic changes in tone, or that the effect of such changes, if they occur, is less important in determining the pulmonary arterial pressure than is the effect of mechanical factors alone.

There is, however, considerable evidence to suggest that acute hypoxia increases the pulmonary vascular tone (16–20). This stimulus raises the pulmonary arterial pressure by a greater amount than might be expected to result from the increase in blood flow which occurs. Moreover, the pulmonary wedge and systemic pressures are not altered (19), and there is no consistent variation in the central blood volume (19, 21). From these observations it may be inferred that hypoxia constricts the vessels of the lungs.

Active dilatation of these vessels has not been so adequately demonstrated. Most drugs which lower the pulmonary arterial pressure have a concomitant action on the systemic pressure, and it is difficult to determine whether the changes in the pulmonary circulation represent a primary or a secondary effect. However, one of us (P.H.) observed that a single dose of acetylcholine can lower the pulmonary arterial pressure without affecting the pressure in the systemic vessels (22, 23). The response was transient, and was found only in patients with a moderate degree of pulmonary hypertension.

Since it seemed possible that this fall in pressure occurred as a result of active vasodilatation, the present project was undertaken with two objectives in view: 1) to investigate the effect of a continuous infusion of acetylcholine into the pulmonary artery of normal subjects, and 2) to inquire whether the action of the drug was enhanced when the pulmonary arterial pressure in these same subjects had been raised by making them hypoxic.

METHODS

Each subject was studied in the unanesthetized basal state. Respiratory studies were carried out on a previous day to acquaint the subject with the laboratory personnel and to accustom him into the effects of breathing a low oxygen mixture.

Catheterization of the pulmonary artery was accomplished in the usual way (24, 25). The position of the catheter was adjusted so that the tip lay just beyond the pulmonic valve. In those subjects in whom the wedged pressure was also measured, a special double-lumen catheter was used which allowed the tip to be wedged while the proximal lumen opened into the main pulmonary artery. With the catheter in place, a cannula was introduced into the right brachial artery. The subject was then allowed to rest quietly for 20 minutes before observations were begun.

Each of the subjects breathed through a mouthpiece for two periods of 20 minutes, separated by a rest of 15 minutes. During one period 21 per cent oxygen was administered, and during the other, a mixture of 12 per

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[2] Fellow of the New York Heart Association.

[3] Fellow of the Nuffield Foundation.

[4] Postdoctoral Research Fellow, United States Public Health Service.

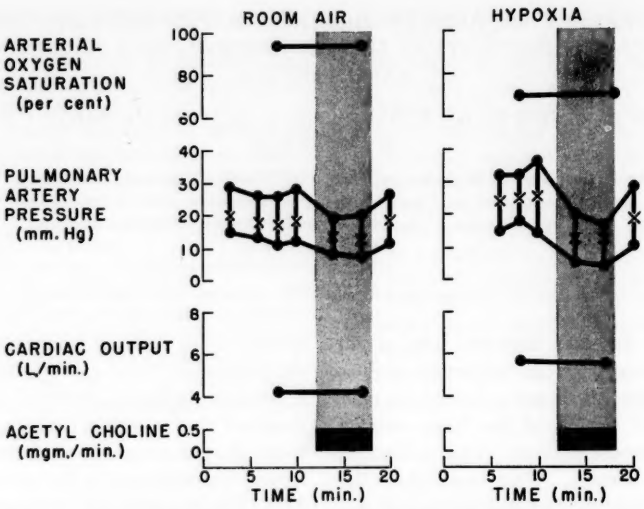

FIG. 1. TIME-COURSE OF CHANGES IN ARTERIAL OXYGEN SATURATION, PULMONARY ARTERIAL PRESSURE, AND CARDIAC OUTPUT IN SUBJECT A.B.

cent oxygen in nitrogen. In alternate patients this sequence was reversed.

Measurements of pulmonary and brachial arterial pressures, and, in three cases, pulmonary wedge pressures, were recorded at the third, sixth, eighth, and tenth minutes of each breathing period. Between the eighth and tenth minutes, a measurement of cardiac output was also made. From the eleventh minute onward, acetylcholine was infused into the main pulmonary artery at the rate of 0.5 mg. per minute. A dilution of 100 mg. acetylcholine per 100 ml. saline was used in order that the volume rate at which saline entered the pulmonary artery during the administration of the drug would equal the rate at which saline was infused during the first 10 minutes to maintain the patency of the catheter. Hence, the possibility that the saline *per se* had an effect was eliminated.

Pressures were recorded at the second and fourth minutes after the start of the infusion, and the cardiac output was remeasured immediately after this second pressure had been obtained. This time-sequence, which was rigidly adhered to in all studies, is illustrated in Figure 1.

The pressures were recorded with a multi-channel cathode ray oscillographic recorder, using Statham gauges as pressure transducers. Average systolic and diastolic pressures were calculated over at least two respiratory cycles, and mean pressures were obtained by planimetric integration.

While the subject breathed 21 per cent oxygen, the cardiac output was estimated by the Fick principle. The oxygen and carbon dioxide contents of the arterial and mixed venous blood samples were determined directly, using the method of Van Slyke. Gas samples were analyzed using a Scholander microanalyzer.

Under hypoxia, the cardiac output was measured by the Stewart-Hamilton dilution principle (26). This method was thought to be preferable to the direct Fick since insufficient time was allowed for the establishment of a steady state of the pulmonary gas exchange (18). The technique entailed injecting 3 ml. of Evans blue dye through the catheter into the main pulmonary artery. A dilution curve was inscribed by sampling blood from the brachial artery through a recording densitometer (27). The densitometer was calibrated by using the pooled-sample method of McNeely and Gravallese (28).

TABLE I

Physical characteristics of the subjects studied

Subject	Sex	Age	Weight	Body surface area
			Kg.	M^2
		Group A		
A. C.	F	30	72.5	1.78
A. B.	F	32	46.6	1.46
J. M.	M	33	45.0	1.46
C. M.	M	33	77.0	1.99
J. P.	M	30	77.5	1.97
W. vP.	M	43	64.8	1.79
H. R.	F	29	61.6	1.70
R. J.	M	32	70.0	1.81
		Group B		
J. A.	M	59	64.0	1.78
W. P.	F	33	65.8	1.75
J. D.	M	39	73.0	1.84
J. G.	M	25	66.2	1.80
P. M.	M	35	51.6	1.60

Dye concentrations in serum were read in a Beckman Model DU spectrophotometer.

The "central blood volume" was calculated by the method of Hamilton, Moore, Kinsman, and Spurling (26). This calculated value presumably included the volume of blood in the pulmonary vessels, in the chambers of the left heart, and in those segments of the systemic arterial tree which lay at the same temporal distance from the root of the aorta as the right brachial artery.

Subjects. The physical characteristics of the subjects are listed in Table I. Each was a convalescent patient who was believed to have a normal pulmonary circulation. The eight subjects in Group A were studied while breathing both 21 and 12 per cent oxygen, while those in Group B were studied while breathing 21 per cent oxygen alone.

Subjects J. P. and W. vP. had had pneumonia, but had been asymptomatic for two weeks prior to the day the study was performed. In both, the abnormal X-ray shadows in the lungs had disappeared. Subject J. A. had been admitted to the hospital for treatment of nontuberculous empyema, but had been symptom-free for a period of six months. Subject R. J. exhibited a moderative degree of anemia.

RESULTS

The data presented in Tables II and III indicate that while 21 per cent oxygen was breathed the results recorded in Groups A and B were similar. In the interest of simplifying the pre-

TABLE II

Effect of acetylcholine on gas exchange and blood gas composition while subjects breathed 21 per cent oxygen *

Subject	Infusion of acetylcholine	$\dot{V}_E$	$\dot{V}_{O_2}$	R_E	CAP_{O_2}	Ca_{O_2}	$(Ca_{O_2}-C\bar{v}_{O_2})$
		L./min./M.²	*ml./min./M.²*		*ml./100 ml.*	*ml./100 ml.*	*ml./100 ml.*
			Group A				
A. C.	Before	4.02	134	0.80	15.9	15.2	4.0
	During	4.22	135	0.76	15.7	14.9	4.1
A. B.	Before	5.48	103	0.95	15.0	13.5	3.6
	During	4.93	102	0.93	15.0	13.5	3.6
J. M.	Before	4.60	138	0.92	19.2	18.1	3.7
	During	4.51	131	0.96	19.2	17.8	3.4
C. M.	Before	3.45	153	0.76	20.8	19.5	3.7
	During	3.67	148	0.83	20.8	19.4	3.6
J. P.	Before	4.42	161	0.78	15.9	15.0	3.9
	During	4.78	164	0.78	15.9	15.2	4.0
W. vP.	Before	6.29	147	0.76	16.7	15.4	4.3
	During	6.06	138	0.77	16.7	15.7	4.8
H. R.	Before	4.20	121	0.63	14.3	13.1	3.6
	During	4.37	142	0.70	14.3	13.1	3.6
R. J.	Before	4.49	138	0.81	12.5	11.7	2.8
	During	5.19	160	0.81	12.7	11.8	3.0
			Group B				
J. A.	Before	3.91	117	0.80	16.3	15.6	3.9
	During	4.60	129	0.83	16.1	15.3	3.6
W. P.	Before	3.38	97	0.80	14.8	13.8	3.5
	During	3.23	93	0.82	14.6	13.7	3.4
J. D.	Before	4.16	122	0.82	19.3	18.0	4.2
	During	4.23	122	0.81	19.2	17.7	4.1
J. G.	Before	3.73	122	0.85	22.4	20.8	4.2
	During	3.97	146	0.74	22.6	21.0	4.4
P. M.	Before	4.26	134	0.87	16.8	15.3	3.7
	During	4.08	131	0.79	16.6	15.2	4.0

* The symbols used in Tables II, III, and IV are defined as follows (63): $\dot{V}_E$, volume of gas expired per minute (BTPS). $\dot{V}_{O_2}$, volume of oxygen taken up per minute (STPD). R_E, respiratory exchange ratio. $F_{I_{O_2}}$, fraction of oxygen in the inspired air. Sa_{O_2}, arterial blood oxygen saturation. CAP_{O_2}, arterial blood oxygen capacity. Ca_{O_2}, arterial blood oxygen content. $C\bar{v}_{O_2}$, mixed venous blood oxygen content.

TABLE III

Effect of acetylcholine on arterial oxygen saturation, heart rate, cardiac index, central blood volume and pressures in the pulmonary and systemic circulations while subject breathed 21 and 12 per cent oxygen

Subject	F_{IO_2}	Infusion of acetylcholine	Sa_{O_2}	Heart rate	Cardiac index	Central blood volume	Pulmonary arterial pressure*			Wedge pressure	Brachial arterial pressure*		
			%		*L./min./M.²*	*L./M.²*	*mm. Hg*			*mm. Hg*	*mm. Hg*		
					Group A		S.	D.	M.		S.	D.	M.
A. C.	0.21	Before	98	90	3.3		18	8	12		146	95	116
		During	98	89	3.3		16	7	11		140	91	115
	0.12	Before	71	108	4.6	0.637	36	16	24		131	87	108
		During	67	104	4.8	0.676	22	10	15		134	91	111
A. B.	0.21	Before	93	61	2.9		27	12	18		108	65	83
		During	93	57	2.8		20	8	13		103	65	82
	0.12	Before	69	71	3.8	0.665	34	16	25		104	70	82
		During	70	78	3.8	0.640	18	5	12		100	59	77
J. M.	0.21	Before	96	72	3.7		21	6	11	9	123	75	94
		During	95	72	3.8		19	6	11	10	121	71	94
	0.12	Before	75	89	4.7	0.740	24	7	16	10	120	68	89
		During	68	90	6.6	1.200	24	7	15	10	124	68	88
C. M.	0.21	Before	95	81	4.1		25	12	18	9	145	85	113
		During	95	76	4.1		23	11	16	9	141	85	108
	0.12	Before	70	93	5.6	0.837	33	15	23	6	145	87	110
		During	64	91	5.5	0.802	26	12	20	7	146	87	111
J. P.	0.21	Before	97	78	4.1		13	5	10		134	79	98
		During	98	80	4.1		12	5	9		137	83	103
	0.12	Before		85	6.2	0.857	18	8	12		146	83	103
		During	78	82	6.8	0.854	14	7	11		142	79	100
W. vP.	0.21	Before	95	73	3.4		30	13	20		150	94	113
		During	96	71	2.9		25	11	17		150	92	117
	0.12	Before		86	5.9	0.855	36	15	24		149	91	117
		During	76	81	6.5	0.989	33	13	22		146	85	112
H. R.	0.21	Before	94	81	3.4		20	9	14	7	112	72	93
		During	94	90	3.9		19	8	13	7	116	75	93
	0.12	Before	77	104	4.9	0.608	24	12	17	5	113	75	89
		During	81	113	5.3	0.644	21	11	14	5	111	74	88
R. J.	0.21	Before	96	76	5.0		26	13	19		130	84	100
		During	96	75	5.3		25	12	17		133	85	102
	0.12	Before	82	83	4.6	1.159	29	15	21		123	80	95
		During	79	80	5.8	0.910	23	11	17		127	81	97
					Group B								
J. A.	0.21	Before	98	70	3.0		28	12	18		125	78	100
		During	97	73	3.6		30	13	19		131	86	104
W. P.	0.21	Before	96	76	2.8		15	5	11		123	85	103
		During	96	84	2.7		14	4	10		119	82	99
J. D.	0.21	Before	95	78	2.9		15	6	10		109	71	91
		During	94	76	2.9		6	3	4		115	75	91
J. G.	0.21	Before	94	97	2.8		19	9	14		126	77	95
		During	95	101	3.3		19	10	14		128	81	97
P. M.	0.21	Before	93	86	3.6		27	11	18		153	95	119
		During	94	88	3.3		24	10	17		154	95	122

* S., systolic; D., diastolic; M., mean.

sentation of the data, therefore, only the results obtained in the eight subjects in Group A will be discussed. As previously noted, these eight subjects were studied while breathing both 21 and 12 per cent oxygen. The effects of acetylcholine at both levels of oxygenation are summarized in Table IV.

TABLE IV

Summary of the average values obtained in the eight subjects in group A *

	F_{IO_2}	Before infusion	During infusion
Ventilation (L./min./M.²)	0.21	4.62	4.72
	0.12		
Arterial oxygen saturation (%)	0.21	96	96
	0.12	74†	72†
Heart rate	0.21	77	76
	0.12	90	90
Cardiac index (L./min./M.²)	0.21	3.74	3.78
	0.12	5.04	5.64
"Central blood volume" (L./M.²)	0.21		
	0.12	0.795	0.839
Pulmonary arterial pressure (mm. Hg)	0.21	23/10 (15)	20/9 (13)
	0.12	29/13 (20)	23/10 (16)
Pulmonary wedge pressure (mm. Hg)‡	0.21	8	9
	0.12	7	7
Brachial arterial pressure (mm. Hg)	0.21	131/81 (101)	130/81 (101)
	0.12	129/80 (99)	129/78 (98)

* With the exception of the Ventilation, Cardiac index, and "Central blood volume," the average values have been rounded off to the nearest whole number.
† Measured in six subjects.
‡ Measured in three subjects.

A. Pressure in the pulmonary artery

Since several factors were operative which might alter the pulmonary arterial pressure, the experimental protocol was set up according to the factorial design devised by Fisher (29). The data, therefore, have been analyzed by the analysis of variance. This approach allows the questions posed earlier to be answered; namely, 1) does acetylcholine affect the pulmonary arterial pressure, and 2) is the effect more pronounced during hypoxia?

The results of the analyses are included in the Appendix. They indicate that the fall in the pulmonary arterial mean pressure produced by acetylcholine would have arisen by chance less frequently than 1 in 20 times ($F = 12.18$, $0.05 > p > 0.01$). They further indicate that the heightened response during hypoxia would also have arisen by chance less frequently than 1 in 20 times ($F = 7.15$, $0.05 > p > 0.01$). Separate analyses of these data by the simpler "t" test showed effects of the same order of magnitude, both when breathing ambient air ($t = 2.9396$, DF (degrees of freedom) $=7$, $0.05 > p > 0.02$) and under hypoxia ($t = 3.3304$, DF $= 7$, $0.02 > p > 0.01$). In all these analyses, the two measurements made immediately before the infusion was

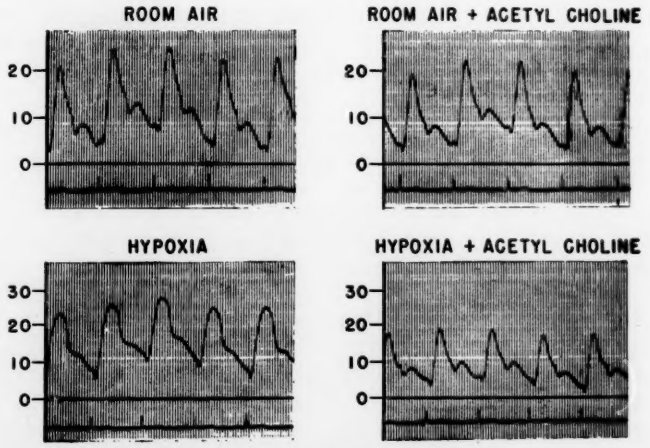

FIG. 2. INFLUENCE OF HYPOXIA AND ACETYLCHOLINE ON THE PRESSURE PULSE RECORDED FROM THE PULMONARY ARTERY

begun were compared with the two made after the infusion had been started.

As can be seen in Table III, the effect of acetylcholine on the systolic and diastolic pressures was similar to the effect on the mean pressure. Of particular interest is the fall in systolic pressure produced by the drug when the subjects breathed ambient air. This fall would have arisen by chance less frequently than 1 in 100 times ($F = 13.06$, $p < 0.01$).

It is noteworthy that in three subjects acetylcholine altered the contour of the pressure pulse recorded from the pulmonary artery. An example is shown in Figure 2. It can be seen that hypoxia produced a rounding of the systolic peak such as is seen in vasoconstriction. With the administration of acetylcholine, the characteristics of the pulse contour returned to those observed before the application of the hypoxic stimulus.

B. Pulmonary wedge pressure

Because the experimental protocol necessitated measuring several variables within a limited period of time, it was not possible to secure records of wedge pressure in all of the studies. However, in three subjects simultaneous tracings of wedge and pulmonary arterial pressures were recorded by using the double-lumen catheter described under Methods. In none of these subjects was the wedge pressure affected by either hypoxia or acetylcholine.

C. Cardiac output

While breathing 21 per cent oxygen, the average cardiac index was 3.74 L. per minute per M.^{2} before the drug was administered, and 3.78 L. per minute per M.^{2} during the period of the infusion. An analysis of these data indicated that the difference between these mean values could easily have arisen by chance ($t = 0.3631$, $DF = 7$, $p > 0.5$).

After 12 minutes of hypoxia, the average cardiac index had increased to 5.04 L. per minute per M.^{2}. During the infusion, the average was 5.63 L. per minute per M.^{2}. This difference between the cardiac indices measured before and during the infusion would have arisen by chance less frequently than 1 in 20 times ($t = 2.5432$, $DF = 7$, $0.05 > p > 0.02$). In every subject the cardiac output either remained unchanged or increased slightly while the drug was being infused. In only two subjects (J. M. and R. J.) did this change in output exceed 10 per cent, the figure usually given as the reproducibility of this measurement under basal conditions.

Thus, the fall in pulmonary arterial pressure during the administration of acetylcholine could not be ascribed to a reduction in pulmonary blood flow.

D. Heart rate

There was no evidence that acetylcholine affected the heart rate. While the subjects breathed 21 per cent oxygen the average rate was 76.5, and during the infusion of acetylcholine, 76.3. Although with hypoxia the average rate increased to 89.9, an identical value was recorded during the infusion.

E. Pressure in the brachial artery

As can be seen in Table IV, the average systolic, diastolic, and mean pressures in the brachial artery were not altered by either hypoxia or acetylcholine.

F. Central blood volume

The effect of acetylcholine on the central blood volume was studied only during hypoxia. There was no consistent pattern of change. The average value before infusion was 0.795 L. per M.^{2}, and during the infusion 0.839 L. per M.^{2}. This difference could have arisen by chance ($t = 0.6300$, $DF = 7$, $p > 0.5$).

It should be emphasized that this is a crude measurement, and that these results do not rule out the possibility that small changes in volume occurred. For instance, in a previous study, two successive measurements of the central blood volume were made under basal conditions in eight normal subjects (21). It was found that the standard deviation of the differences between each pair of measurements was 9 per cent of the mean. It was also established that acute hypoxia did not produce alterations which lay beyond this range.

G. Arterial oxygen saturation

Acetylcholine did not change the arterial oxygen saturation while the subjects breathed 21 per cent oxygen. The maximum difference observed in any single subject was only 1 per cent. The average values of saturation before and during the infusion were 95.5 and 95.6 per cent, respectively.

At the end of 12 minutes of hypoxia, the average saturation of the six subjects in whom it was measured had fallen to 74 per cent. During the period of infusion the average saturation in these same subjects was 71.5 per cent. Thus, the fall in the pulmonary arterial pressure during the infusion could not be ascribed to a lessening of the hypoxic stimulus.

H. Ventilation

The ventilation was measured only while 21 per cent oxygen was breathed. Acetylcholine had no apparent effect. Before the drug was administered the average ventilation was 4.62 L. per minute per M.^{2}, and during the infusion 4.72 L. per minute per M.^{2}.

DISCUSSION

Effect of acetylcholine on the pulmonary vessels

The recognition of vasomotor activity depends on the interpretation of alterations in resistance. This resistance is calculated by dividing the pressure drop across a segment of the circulation by the volume rate at which it is perfused with blood. But, in addition to the vascular tonus, this ratio depends on several other factors, notably the rate of flow, the pressures at the ends of the system, and the composition of the blood itself (30–36). Hence, any change in resistance must be interpreted with caution. On the one hand, it may indicate vasomotor activity. On the other, it may simply reflect a variation in one of the associated factors.

In the pulmonary circulation the situation is even further complicated by the variability of the pressure outside the vessels and by the fact that the volume of blood in the lungs can be altered by systemic vasocontriction or dilatation. For all of these reasons it would seem to be impossible to estimate changes in vasomotor tone in any exact way. However, one can be reasonably certain that vasodilatation has occurred if the pulmonary arterial pressure falls in the face of the following conditions: 1) a constant or increased cardiac output; 2) a constant pressure in the left atrium; 3) an unchanged extravascular pressure within the lungs and thorax; and 4) a constant heart rate, systemic pressure, and pulmonary blood volume.

Using these criteria, an examination of our data reveals the following points. Acetylcholine lowered the pulmonary arterial pressure while the cardiac output either remained constant or increased. In the three subjects in whom an assessment of left atrial pressure was obtained by using the wedged catheter technique, no change in this pressure was observed. Although the extravascular pressure within the lungs and thorax was not measured, there was no evidence that any change took place; for instance, the minute volume of ventilation and the respiratory frequency remained constant, and no respiratory symptoms developed. Finally, the heart rate and systemic blood pressure were not altered, and the central blood volume, which includes the volume of blood in the pulmonary vessels, showed no consistent variation. These observations, which are in conformity with limited results previously reported (37, 38), suggest that acetylcholine dilated the vessels of the lungs.

The mechanism whereby the effect of acetylcholine is mediated

On the basis of these data, it may be presumed that the drug in some way acted on the muscle fibers of the medial coats of the small branches of the pulmonary vascular tree. Theoretically, it might have affected these fibers directly, reaching them either by diffusion through the vessel wall or via the vasa vasorum from the bronchial circulation. The possibility that diffusion through the wall occurred seems plausible, since it has been demonstrated that acetylcholine can alter the tone of vascular rings *in vitro* (39, 40).

If instead the drug reached the pulmonary vessels by recirculation through the bronchial arteries, it might have been expected to have caused vasodilatation elsewhere in the systemic circula-

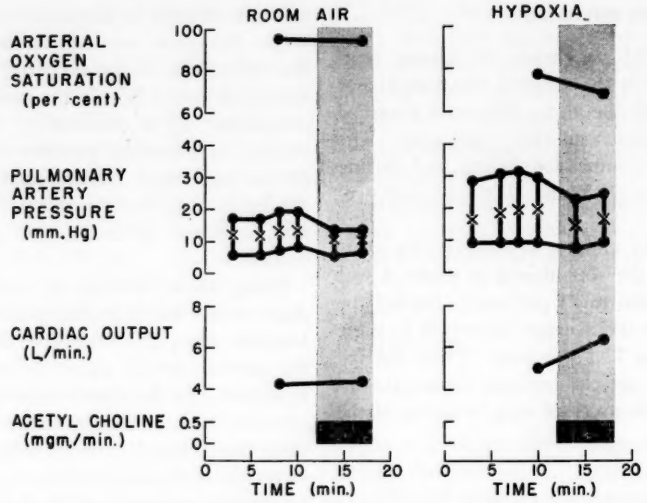

FIG. 3. TIME-COURSE OF CHANGES IN ARTERIAL OXYGEN SATURATION, PULMONARY ARTERIAL PRESSURE AND CARDIAC OUTPUT IN SUBJECT E.B. WHO HAD HAD A TOTAL SYMPATHECTOMY

tion or to have produced bronchoconstriction. No such effects were evident.

Alternatively, the action of the drug may have been transmitted by means of nervous pathways. One such possibility can be ruled out on the basis of a study performed on a subject who had had a complete surgical sympathectomy. The results are shown in Figure 3. Fishman, Himmelstein, Fritts, Lahoz, and Cournand (20) have previously reported that hypoxia can produce pulmonary hypertension in a patient in whom the cervical and upper four sympathetic ganglia have been resected. The present results suggest that both the effect of hypoxia and of acetylcholine can be mediated with all of the sympathetic ganglia removed.

Effect of hypoxia on the response to acetylcholine

The influence of acetylcholine was in general more apparent during hypoxia than when the same subjects breathed room air. It is noteworthy, also, that this increased action of acetylcholine under hypoxia was most striking in those patients in whom the effect of hypoxia on the pulmonary circulation was greatest. Hence, it might be suspected that hypoxia and acetylcholine are in some way synergistic.

But in an earlier study one of us observed that a single dose of acetylcholine had the same marked effect in patients who had developed pulmonary hypertension as a consequence of heart disease (*22, 23*). The effect was transient, and was not evident in patients in whom the pulmonary arterial pressure was normal. To explain these observations it was suggested that the response depends on the preexisting vascular tone and the thickness of the media of the small branches of the pulmonary artery.

Since there is considerable evidence to indicate that hypoxia constricts the vessels of the lungs (16–20, 41, 42), it seems likely that an augmented tone is the common factor in the previous and the present results. There does not appear to be any evidence that hypoxia *per se* potentiates the effect of acetylcholine.

Results reported by others

Finally, the results of the present study must be examined in the light of those which have been reported by others. Most of the experiments have

been carried out on laboratory animals (43–56). A review of these papers indicates that no consistent pattern of response was obtained.

In man, intravenous injections of acetylcholine have been shown to cause vasodilatation in the systemic circulation. Villaret and Justin-Besançon (57), Ellis and Weiss (58, 59), and Carmichael and Fraser (60) all observed that intravenous doses of the drug produced either a fall in blood pressure or a flushing of the skin. Ellis and Weiss (58) fixed the minimal effective infusion rate as lying between 50 and 60 mg. per minute. It should be pointed out that this is from 80 to 120 times greater than the dosage used in the present study. It is believed, therefore, that with the infusion of 0.5 mg. per minute the effect of the drug was largely dissipated before it reached the left side of the heart and the systemic circulation.

This belief is supported by the results of Duff, Greenfield, Shepherd, and Thompson (61) who investigated the rapidity with which cholinesterase inactivates acetylcholine in the bloodstream. Using a plethysmograph to measure changes in blood flow, they demonstrated that as little as 0.00025 mg. acetylcholine would augment the flow through the forearm when injected into the brachial artery. The dose had to be increased about one thousand fold in order to augment the blood flow through the hand. They attributed this phenomenon to the inactivation of the drug by the enzyme. Moreover, they found that when acetylcholine was incubated with blood for 10 seconds before the injection, the potency of the dose was diminished by more than 99.7 per cent of its original strength. These results confirm the early observations of Tiffeneau and Beauvallet (62), who demonstrated that, once introduced into the cardiovascular system, the effect of acetylcholine is rapidly neutralized.

Conclusions

It is tempting to say that our results indicate that acetylcholine, a naturally occurring substance, plays a role in controlling the pulmonary arterial pressure in normal man. But this conclusion is not justified. Pharmacologic, not physiologic, doses have been used and marked responses have been observed only when the pulmonary arterial pressure was elevated. Thus it may be that acetylcholine plays a part in regulating the pulmonary circulation in disease states in which pulmonary hypertension exists.

From a practical standpoint, the data suggest that acetylcholine or a related substance of longer action might be useful in the therapy of patients with acute or chronic pulmonary hypertension. The drug might also provide information regarding the status of the pulmonary vessels of patients in whom pulmonary hypertension has been present for a prolonged period of time.

SUMMARY

Acetylcholine has been infused into the pulmonary artery of normal human subjects under normal and hypoxic conditions. The drug caused a fall in pulmonary arterial pressure which was more evident after hypoxia had produced pulmonary hypertension. The fall in pressure was not associated with a fall in cardiac output, and there was no change in the pulmonary wedge pressure, heart rate, systemic blood pressure, or central blood volume.

It is concluded that acetylcholine causes pulmonary vasodilatation. Apparently the effect is enhanced in the presence of an increased vascular tone.

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APPENDIX

Analysis of variance of mean pulmonary arterial pressure in the eight subjects of group A

Source of variance	$S(X-\bar{X})^2$	DF	Variance
Total	1257.4	63	
Subjects	630.4	7	90.06
Acetylcholine	168.9	1	168.9
$F_{I_{O_2}}$	210.2	1	210.2
Subjects × acetylcholine	97.1	7	13.87
Subjects × $F_{I_{O_2}}$	63.3	7	9.043
Acetylcholine × $F_{I_{O_2}}$	39.1	1	39.1
Subjects × acetylcholine × $F_{I_{O_2}}$	38.3	7	5.471
Residuum	10.1	32	0.3156

Analysis of variance of systolic pulmonary arterial pressure in the eight subjects of group A

Source of variance	$S(X-\overline{X})^2$	DF	Variance
Total	2615.4	63	
Subjects	1357.5	7	193.9
Acetylcholine	337.7	1	337.7
F_{IO_2}	375.4	1	375.4
Subjects×acetylcholine	181.0	7	25.86
Subjects×F_{IO_2}	166.8	7	23.83
Acetylcholine×F_{IO_2}	62.0	1	62.0
Subjects×acetylcholine×F_{IO_2}	88.9	7	12.7
Residuum	46.1	32	1.44

Analysis of variance of diastolic pulmonary arterial pressure in the eight subjects of group A

Source of variance	$S(X-\overline{X})^2$	DF	Variance
Total	709.4	63	
Subjects	355.4	7	50.77
Acetylcholine	85.5	1	85.5
F_{IO_2}	68.0	1	68.0
Subjects×acetylcholine	84.5	7	12.07
Subjects×F_{IO_2}	36.5	7	5.214
Acetylcholine×F_{IO_2}	22.6	1	22.6
Subjects×acetylcholine×F_{IO_2}	50.5	7	7.214
Residuum	6.4	32	0.2

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VENTILATORY MECHANICS IN PULMONARY EDEMA IN MAN [1]

By JOHN TURNER SHARP,[2] GERAINT T. GRIFFITH,[3] IVAN L. BUNNELL,[4] AND DAVID G. GREENE

(From the Department of Medicine of the University of Buffalo School of Medicine and the Buffalo General Hospital)

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The mechanics of breathing have recently been investigated in patients with a variety of pulmonary abnormalities including pulmonary congestion due to heart disease (1–3). The present report describes ventilatory mechanics in eight patients with frank pulmonary edema. The data were calculated from simultaneous oscillographic records of tidal volume, air-flow, and esophageal pressure.

CLINICAL MATERIAL

Eight patients were studied while in pulmonary edema, and seven of the eight patients were restudied following recovery from pulmonary edema. The clinical criteria for the diagnosis of pulmonary edema were as follows: 1) moderate to marked dyspnea at rest, 2) moist rales over at least one-half of both lung fields in the absence of fever or other signs of pulmonary infection, 3) evidence of incapacitating heart disease with previous episodes of pulmonary congestion or edema, 4) roentgenographic changes consistent with those seen in pulmonary edema where chest roentgenograms were available. In all cases except Case 7, rales were still present during the recovery study indicating the presence of pulmonary congestion. Three patients had rheumatic valvular heart disease, three had arteriosclerotic heart disease, one had hypertensive cardiovascular disease and the remaining patient had myocardial fibrosis and abscess formation and severe anemia. Only in Case 2 was there evidence for any other lung disease than pulmonary edema and congestion. A brief clinical account of each patient is presented in the Appendix.

METHODS

Intraesophageal pressure was recorded by means of a 60 cm. long polyethylene tube with an internal diameter of 0.09 cm.[5] filled with boiled water and attached to a pressure transducer of low volume displacement.[6] This system gave a 95 per cent response to a square wave within 0.02 seconds. The natural frequency was 17 cycles per second. Care was taken that the tip of the esophageal tube was in the middle or upper third of the esophagus. The fluid-filled tube was used instead of an air-filled balloon because its small size enabled it to be swallowed more easily by acutely dyspneic patients. Air-flow was measured by recording the differential pressure [7] across a 400 mesh, monel-metal screen 2 inches in diameter. Tidal volume was measured in a carefully balanced spirometer.[8] To the spirometer counter-weight was attached an electrical contact to a resistance wire which formed two arms of a resistance bridge. This bridge was activated by a mercury cell and the output fed into a DC amplifier. The output of this volume transducer was linearly related to volume up to the capacity of the spirometer bell (9 liters). Rebreathing of expired air occurred in using this spirometer. Pressure at the mouth was recorded by another pressure transducer,[9] and for normal breathing was consistently under 1.5 cm. of water. This pressure, representing the frictional and inertial resistance of the pneumotachometer and spirometer, was subtracted from esophageal pressure in calculating resistance. Esophageal pressure, air-flow, tidal volume, and mouth pressure tracings were recorded photographically as shown in Figure 1.

Lung compliance, expressed in liters per cm. of water (L. per cm. H_2O), was obtained by dividing the tidal volume by the change in the esophageal pressure between points of zero flow. Resistance was calculated at points of 1 L. per second air-flow by the method of von Neergaard and Wirz (4). In this method, the volume (above end-tidal volume), measured at the point at which resistance was to be determined, was divided by the compliance to yield the pressure which would be present in the esophagus if no air were flowing. The difference between this calculated "static pressure" and the pressure actually measured is the additional pressure necessary to overcome the resistance of airways, tissues

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[2] Established Investigator of the American Heart Association.

[3] Junior Board Fellow of the Buffalo General Hospital.

[4] Supported by the John A. Hartford Foundation.

[5] Clay-Adams Co., PE 100.

[6] Statham P23 Ga pressure transducer.

[7] Statham P5 0.2 p.s.i.d. pressure transducer.

[8] Warren E. Collins Co. respirometer.

[9] Statham P23 D pressure transducer.

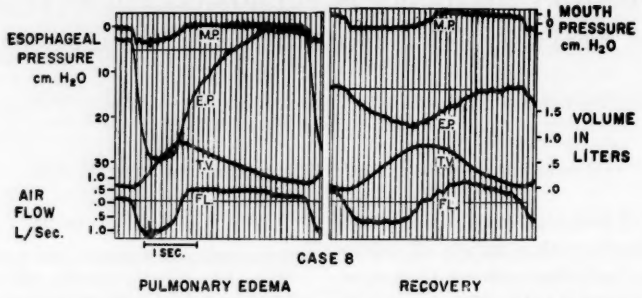

FIG. 1. OSCILLOGRAPHIC RECORD OF MOUTH PRESSURE (M.P.), ESOPHAGEAL PRESSURE (E.P.), TIDAL VOLUME (T.V.) AND AIR-FLOW (FL.) DURING PULMONARY EDEMA AND FOLLOWING RECOVERY

and apparatus. Mouth pressure (apparatus resistance) is then subtracted, leaving airway and tissue resistance. Assumption of a linear static volume-pressure relationship for the lung is necessary in order for these calculations of resistance to be valid. Ten of 20 breaths from each study were analyzed for compliance and resistance calculations.

In Cases 1, 4, 6 and 7, air-flow rose above 1 L. per second early in inspiration and fell below it again late in inspiration, so that resistance at 1 L. per second flow could be calculated at a point early in inspiration and at a second point late in inspiration. These early and late inspiratory resistances are compared in Table II and are averaged to give the single value for inspiratory resistance shown in Table I. In the remaining cases air-flow reached or exceeded 1 L. per second for only a short period during the middle of inspiration. In these cases inspiratory resistance was calculated at a single point.

Resistance has also been calculated at intervals throughout the respiratory cycle and plotted against time as in Figure 2. The resistances shown have been calculated at varying rates of air-flow by dividing the calculated resistance pressure gradient by flow. The peak resistance values obtained in early inspiration were also measured at air-flow rates other than 1 L. per second.

RESULTS

Compliance

As can be seen from Table I, compliance was found to be extremely low in pulmonary edema. The average value was 0.037 L. per cm. H_2O with values ranging from 0.017 to 0.057 L. per cm. H_2O. These values for compliance are generally considerably lower than those reported in patients with pulmonary congestion due to heart disease. Following recovery from pulmonary edema the average compliance was 0.089 L. per cm. H_2O with values ranging from 0.044 to 0.190 L. per cm. H_2O. These values for compliance correspond to values reported by others in patients with pulmonary congestion, and there was clinical evidence of pulmonary congestion in all but Case 7 at the time of follow-up study. All values at the time of frank pulmonary edema are considerably below the average normal compliance of 0.165 L. per cm. H_2O (5). It will be noted that except in Case 6, compliance more than doubled on recovery from pulmonary edema. The values of the standard errors indicate that all differences in compliance in individual patients between pulmonary edema values and recovery values are highly significant ($p < 0.01$).

It is known that in the presence of airway obstruction, compliance falls as the respiratory rate rises (6). The average respiratory rates of the group during pulmonary edema and following recovery, respectively, were 26 and 22. The greatest individual change was from 25 to 19 breaths per minute. It is unlikely that such small changes in breathing frequency could account for compliance change of the magnitude measured.

Resistance

Resistance was significantly increased in pulmonary edema averaging 9.9 cm. H_2O per L. per second during inspiration and 8.7 cm. H_2O per L. per second during expiration. These values are three to four times the normal. Following recovery from pulmonary edema the average resistance during inspiration was found to be 5.3 cm. H_2O per L. per second and during expiration 4.4 cm. H_2O per L. per second. These values are also elevated and are similar to resistance values reported by others (1) in patients with pulmonary

TABLE I

Ventilatory mechanics in eight patients with pulmonary edema

Case no.	Compliance ± S.E. (L./cm. H_2O)		Resistance at 1 L./sec. ± S.E. (cm. H_2O/L./sec.)				Mean tidal volume (L. B.T.P.S.)		Minute volume (L./min. B.T.P.S.)	
			Inspiratory		Expiratory					
	Pulmonary edema	Recovery	Pulmonary edema	Recovery	Pulmonary edema	Recovery	Pulmonary edema	Recovery	Pulmonary edema	Recovery
1	0.017 ± 0.0002	0.044 ± 0.001	10.3 ± 1.0	9.2 ± 0.9	6.7 ± 1.3	3.6 ± 0.4	0.43	0.73	10.4	16.8
2	0.020 ± 0.0006	0.053 ± 0.003	12.7 ± 0.9	3.4 ± 0.3	8.9 ± 0.6	4.4 ± 0.3	0.55	0.38	17.7	9.9
3	0.026 ± 0.002	0.069 ± 0.004	12.1 ± 1.0	9.3 ± 0.9	13.3 ± 1.8	9.1 ± 0.8	0.69	0.74	16.9	13.8
4	0.031 ± 0.0007	0.076 ± 0.0002	9.1 ± 0.5	2.6 ± 0.2	3.9 ± 0.3	3.4 ± 0.2	0.64	1.42	17.9	31.5
5	0.035 ± 0.001		5.9 ± 0.4		11.5 ± 1.2		0.54		15.7	
6	0.052 ± 0.001	0.071 ± 0.001	11.6 ± 1.1	6.8 ± 0.2	11.4 ± 0.7	7.1 ± 0.5	0.82	0.85	18.1	21.3
7	0.054 ± 0.0005	0.190 ± 0.006	7.5 ± 0.6	2.7 ± 0.3	2.8 ± 0.3	0.6 ± 0.08	0.97	0.50	24.5	9.7
8	0.057 ± 0.001	0.120 ± 0.003	10.1 ± 0.3	2.8 ± 0.1	11.1 ± 0.6	2.4 ± 0.2	0.95	0.84	20.0	16.0
Averages ± S.E.	0.037 ± 0.0056	0.089 ± 0.019	9.9 ± 0.83	5.3 ± 1.17	8.7 ± 1.36	4.4 ± 1.09				
p	<0.05		<0.01		<0.05					

TABLE II

Difference between early inspiratory resistance and late inspiratory resistance in four patients

Case no.	Resistance in cm. H_2O at 1 L./sec. flow ± S.E.		
	Early inspiration	Late inspiration	p
	During pulmonary edema		
1	13.7 ± 1.2	6.9 ± 0.6	<0.01
4	10.4 ± 0.7	7.7 ± 0.5	<0.01
6	16.2 ± 0.3	7.1 ± 0.3	<0.01
7	9.7 ± 0.4	5.2 ± 0.2	<0.01
	Following recovery		
1	12.1 ± 0.9	6.2 ± 0.8	<0.01
4	2.4 ± 0.3	2.7 ± 0.3	>0.1
6	7.4 ± 0.3	6.2 ± 0.3	<0.01
7	3.8 ± 0.3	1.6 ± 0.1	<0.01

congestion. In individual cases differences in resistance between pulmonary edema and recovery are significant except for inspiratory resistance in Cases 1 and 3. The differences between the means of pulmonary edema data and recovery data are significant for inspiratory resistance ($p < 0.01$) and for expiratory resistance ($p < 0.05$).

Early inspiratory resistance was consistently and significantly greater than late inspiratory resistance during pulmonary edema in Cases 1, 4, 6 and 7 and, except in Case 4, following recovery from pulmonary edema (Table II). Figure 2 shows resistance plotted against time throughout the respiratory cycle in Case 6. It is seen that inspiratory resistance is highest early in inspiration and falls significantly late in inspiration. The upper curve shows resistance in acute pulmonary

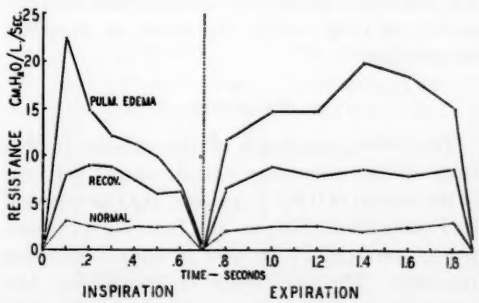

FIG. 2. RESISTANCE PLOTTED AGAINST TIME THROUGH ONE RESPIRATORY CYCLE IN CASE 6

The top curve was obtained during pulmonary edema, the middle one following recovery in the same patient (pulmonary congestion persisted), and the bottom curve from a normal subject.

edema; the middle curve, resistance following recovery when pulmonary congestion was still present; and the lower dotted curve, similar data on a normal subject. In all cases, a peak of resistance was found within the first 0.1 to 0.2 seconds of inspiration. This early inspiratory peak resistance varied from 9.1 to 31.5 cm. of water per L. per second.

The patients studied represent several etiologic types of heart disease. No characteristic relationship between compliance and resistance changes can be associated with the etiologic types of heart disease represented. An attempt was made to determine whether the ratio between compliance change and resistance change is different in patients treated with aminophyllin, a known bronchodilator on the one hand, and morphine, a known bronchoconstrictor, on the other. Three patients (Cases 4, 5 and 7) had neither of these drugs before study, one patient had aminophyllin alone (Case 6), two patients had morphine alone (Cases 2 and 8), and two patients had aminophyllin and morphine (Cases 1 and 3). The small number of patients and the several combinations of treatment limit conclusions concerning the effect of prior treatment with these agents on initial compliance and resistance. However, the absence of outstanding difference between the three cases who had not been treated with these agents prior to study and the remaining cases suggests that any direct effect on lung mechanics which these drugs may have had was a minor one. In all probability the high resistance is a function of pulmonary edema rather than the activity of drugs which are active on bronchial musculature.

DISCUSSION

The mean compliance of the patients in this study during pulmonary edema was 22 per cent of the normal of 0.165 L. per cm. H_2O as reported by Frank, Mead, Siebens, and Storey (5). Compliance averaged 54 per cent of normal following recovery. The occurrence of comparably low values for compliance in experimental pulmonary edema in the dog (7–9) is well documented. That such low compliance values cannot be accounted for by simple replacement of alveolar volume by edema fluid is indicated by simultaneous compliance and lung volume determinations done by Cook, Schreiner, and Mead (9) in dogs with pulmonary edema. These investigators showed that decreases of compliance of 70 per cent were accompanied by decreases in total and ventilated pulmonary gas volumes of only 20 and 32 per cent, respectively. Pulmonary hypertension does not account for such low compliance values as shown by the work of Frank, Radford, and Whittenberger (10) and by the observations of Borst and co-workers (11) where the fall in compliance resulting from left atrial pressures of 50 cm. of water was of the order of 20 to 30 per cent.

An alternative explanation is that a change in either the composition or the geometry of distensible elements of the lungs has occurred to render them less compliant. The principal distensible elements of the alveoli are the elastic fiber network and the alveolar surface film. It is possible that pulmonary edema alters either the properties of elastic fibers or their geometric arrangement. It appears more plausible to the authors, however, that it is the composition and the geometry of the alveolar surface film which is altered in pulmonary edema. The observations of Mead, Whittenberger, and Radford (12) and of Clements (13) indicate that alveolar surface forces play a dominant role in determining lung compliance. Observations of Brown (14) on the surface tension of pulmonary edema fluid suggest that its surface properties are much the same as those of the normal alveolar surface. However if this edema fluid were geometrically arranged as bubbles, the radii of which were smaller than those of the parent alveoli, the resulting compliance would be that of alveoli with radii equal to those of the bubbles.

Assuming that both an alveolus and its contained bubbles are portions of spheres, the volume of such a unit is approximately equal to $4\pi r^3/3$. If one considers only surface compliance, Laplace's law states that transmural pressure is directly proportional to surface tension and is inversely proportional to first power of the radius ($P = 2T/r$). Thus V/P or compliance equals $4\pi r^3/3 \div 2T/r$ or $2\pi r^4/3T$. By application of differential calculus it may be shown that the slope of the volume-pressure curve (dV/dP) as well as the compliance (V/P) varies directly as the fourth power of the radius and inversely as the surface tension. Thus halving the radius would

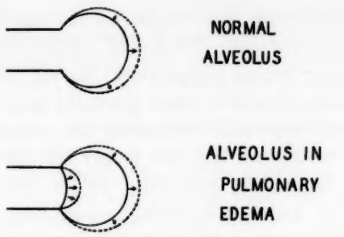

FIG. 3. DIAGRAMMATIC REPRESENTATION OF A NORMAL ALVEOLUS (ABOVE) AND AN ALVEOLUS DURING PULMONARY EDEMA (BELOW) EXPANDING DURING INSPIRATION

The bottom alveolus has at its mouth a bubble of small radius and low compliance.

decrease compliance sixteenfold, while doubling surface tension would only halve compliance. It has been suggested to us by Dr. Elwyn S. Brown that if the majority of alveoli had edema fluid bubbles at their mouths as shown in Figure 3, with inward inspiratory expansion of such bubbles the lung would show the following properties: 1) a striking decrease in compliance would occur accompanied by relatively little decrease in pulmonary gas volume; 2) the ventilated pulmonary gas volume would be less than the total pulmonary gas volume because of trapping of air distal to the bubbles; 3) compliance would be low at the beginning of inflation of the bubble and would rise as the bubbles and their radii of curvature became larger. The work of Cook, Schreiner, and Mead (9) cited above gives evidence that the first two of these effects actually occur in pulmonary edema in the dog. The third effect is also a characteristic of experimental pulmonary edema in the dog as indicated by previous work of the present authors (7) and the work of Whittenberger and Affeldt (15) who have described alinear volume-pressure curves of the lung and thorax similar to that in the right hand portion of Figure 4 in dogs with pulmonary edema.

If one considers the normal resistance not to be above 3 cm. H_2O per L. per second, resistance in pulmonary edema averages more than three times the normal and following recovery averages twice the normal. This increase in resistance is not so difficult to account for as the decrease in compliance considered above. Edema of the airways could account for much of this when one considers that the resistance to laminar flow offered by a tube is inversely proportional to the fourth power of its radius. In addition, foam and secretion in the airways might increase the proportion of turbulent flow under which state the resistance is proportional to the square rather than a first power of flow. These factors would tend to make resistance highest during expiration. This is a prominent feature of resistance-time plots in several of the patients as exemplified in Figure 2. However the high value for resistance seen early in inspiration is not explained fully by a consideration of these factors. One may question whether this inspiratory peak resistance in fact represents true resistance at all. It should be recalled that this resistance is a calculated value and that its calculation involves the assumption that the whole volume-pressure curve within the range of the tidal volume is linear. The lung volume-pressure loops in Figure 4 graphically portray the two situations which might apply. The diagonals within the loops represent the static volume-pressure curves. In order to calculate resistance one assumes the relationship in the left half of the figure in which the static volume-pressure curve is linear throughout the tidal range. The right hand figure is based on the assumption that the actual volume-pressure curve may be curvilinear becoming steeper as inflation continues, as seen in experimental pulmonary edema and as discussed in the preceding paragraph. Both compliance and resistance are lower in the right hand figure at the point early in inspiration at which resistance is being measured. However, the compliances for the two figures are equal when measured over the whole tidal volume.

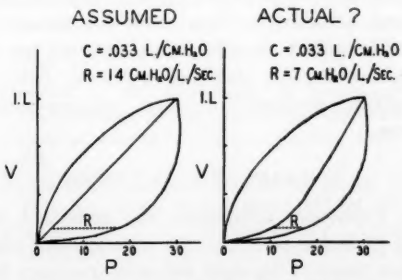

FIG. 4. TOTAL VOLUME-PRESSURE "LOOPS" DURING ONE TIDAL BREATH

The diagonal lines bisecting the loops are the static volume-pressure (compliance) curves, and the right hand limb of each loop is the dynamic inspiratory pressure-volume curve. See text for discussion.

Consequently, overestimation of resistance would occur early in inspiration. Thus it is possible that the same explanation postulated to explain the extremely low compliance and the particular alinear configuration of the lung's static volume-pressure curve in pulmonary edema may also explain the apparent high early inspiratory resistance.

The concept of critical opening pressures of terminal lung units provides another possible explanation of the physical properties of the lungs in pulmonary edema. Burton (16) has pointed out that fine tubular structures such as arterioles are fundamentally unstable and tend to close off if the balance of forces about them is even slightly altered. If tissue edema and fluid in airways narrowed and disturbed the balance of forces about respiratory bronchioles and alveolar ducts causing them to close off, fewer expandable alveoli would be available and compliance would fall. Moreover compliance would be lowest at the beginning of inspiration and would rise progressively with lung inflation as the opening pressures of more and more lung units were exceeded. The apparent high resistance early in inspiration may be a manifestation of high opening pressures of terminal lung units.

A final factor which might account for an additional decrement of compliance is based upon the knowledge that the lung and presumably its individual alveoli become less compliant as they are overdistended. In pulmonary edema, both the uncompliant or fluid-filled alveoli and the normally compliant alveoli are subjected to a greater than normal inflation pressure (greater negative intrapleural pressure). This may overdistend the more compliant alveoli so that they too are less compliant because they are operating over less compliant portions of their volume-pressure curves.

SUMMARY AND CONCLUSIONS

1. Pulmonary compliance and resistance have been studied in eight patients in pulmonary edema and in seven of the eight following recovery from pulmonary edema.

2. Compliance was found to be very low in pulmonary edema, averaging 0.037 ± 0.0056 (S.E.) L. per cm. H_2O or 22 per cent of the average normal value. Following recovery, the average compliance was 0.089 ± 0.019 L. per cm. H_2O.

3. Resistance was found to be markedly increased, averaging 9.9 ± 0.83 (S.E.) cm. H_2O per L. per second (inspiration) during pulmonary edema, a value over three times the normal. On recovery inspiratory resistance fell to an average of 5.3 ± 1.17 cm. H_2O per L. per second.

4. The highest resistance values were found early in inspiration during pulmonary edema. This high early inspiratory resistance may be an indirect expression of alinearity of the static volume-pressure curve of the lung in pulmonary edema rather than true resistance.

5. The effect on compliance of bubbles in alveoli is discussed. It is suggested that the presence of such bubbles at alveolar orifices might account for the disproportionate lowering of compliance with respect to pulmonary gas volume change, the trapping of gas, the alinearity of the static volume-pressure curve and the apparent high early inspiratory resistance found in pulmonary edema.

6. Elevation of the opening pressures of terminal lung units has been suggested as an alternative explanation for the properties of the lungs in pulmonary edema.

APPENDIX

Case 1, A.F., was a 64 year old white female with inactive rheumatic valvular heart disease with both mitral and aortic involvement. Pulmonary edema was precipitated by sodium retention secondary to phenylbutazone which had been given for a tendonitis. When initially studied, moist rales were heard to the level of midscapulae bilaterally, and expiratory wheezes were prominent. The patient was considered to be in acute pulmonary edema. Before study, morphine, mercuhydrin, aminophyllin, oxygen by mask, and tourniquets had been administered to the patient. This patient was studied while in acute pulmonary edema and after recovery two days later.

Case 2, W. C., was a 67 year old white male with a profound macrocytic anemia of unknown cause. Moist rales were heard diffusely over both lung fields and the patient was considered to be in acute pulmonary edema. Morphine had been given approximately three hours previous to the initial study. He was studied while in acute pulmonary edema and again following recovery, four days later. Ten days following the last study the patient expired, and at necropsy in addition to severe recurrent pulmonary edema with myocardial abscesses and fibrosis, he also had pulmonary fibrosis due to silicosis. The low compliance was probably partially due to the silicosis, but it is thought that the changes in compliance and resistance which occurred with recovery represented changes in the degree of pulmonary edema.

Case 3, M.O., was a 54 year old white female with chronic rheumatic heart disease with mitral stenosis. When initially studied, moist rales were present from apices to bases bilaterally, and the patient was considered to be in acute pulmonary edema. The patient had received morphine and aminophyllin 80 minutes prior to the initial study and was on maintenance digitalis. When restudied two days later she was not dyspneic at rest.

Case 4, N.Z., was a 64 year old white male with arteriosclerotic heart disease. Moist rales were heard over the lower halves of both lung fields, and the patient was considered to be in subacute pulmonary edema. He had received digitalis and Demerol® two hours before the initial study and had been treated with intermittent positive pressure, oxygen and tourniquets. He was studied while in acute pulmonary edema and following recovery two days later.

Case 5, F.W., was a 57 year old Negro male with hypertensive cardiovascular disease and uremia. He was producing blood-tinged frothy sputum, and moist rales could be heard bilaterally to the level of the mid-scapulae. He was considered to be in acute pulmonary edema. The patient had not been treated prior to study except for maintenance digitalis. He expired two days after the study and neither autospy nor follow-up study were obtained.

Case 6, F.S., was a 54 year old white male with rheumatic heart disease with mitral stenosis and insufficiency. Moist, bubbling rales were heard throughout both lung fields initially, but by the time he was studied these had improved significantly; therefore he was considered in subacute pulmonary edema. Within an hour prior to study he had been treated with aminophyllin, mercuhydrin, digoxin, positive pressure oxygen and tourniquet administration. He was studied in pulmonary edema and following recovery two days later.

Case 7, E.B., was a 65 year old white male who had arteriosclerotic heart disease. Moist rales were heard throughout both hemithoraces from the level of the clavicles to the bases, and the patient was considered to be in acute pulmonary edema when first studied. No treatment had been given prior to the initial study. The patient was studied while in acute pulmonary edema and again two days later. He expired suddenly three days after the last study and at necropsy was found to have old and recent myocardial infarction and severe pulmonary edema.

Case 8, H.L., was a 58 year old white male with arteriosclerotic heart disease and electrocardiographic evidence of old myocardial infarction. At the time of initial study, coarse bubbling rales were heard diffusely over both lung fields, expiration was prolonged, and wheezes were prominent. He had received morphine approximately three hours prior to study. The patient was studied while in acute pulmonary edema and following recovery three days later.

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THE INFLUENCE OF DIETARY CARBOHYDRATE DEPRIVATION ON THE METABOLISM OF INTRAVENOUSLY ADMINISTERED FRUCTOSE AND GLUCOSE IN MAN [1, 2]

By JAMES W. CRAIG, MAX MILLER, MALCOLM S. MACKENZIE, AND HIRAM WOODWARD, JR.

(From the Department of Medicine, School of Medicine, Western Reserve University, Cleveland, Ohio)

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The phenomenon of starvation diabetes or the induction of impaired carbohydrate tolerance by the restriction of dietary carbohydrate has been recognized for almost a century. Peters (1) reviewed the subject comprehensively in 1945. The effect of carbohydrate deprivation upon the changes in the total blood sugar concentration following fructose administration has been investigated previously in animals (2–4) and in a human subject (5). Since the blood levels of glucose and fructose were not determined separately, it is not possible to ascertain from the results of these studies the effect of carbohydrate deprivation upon the fructose tolerance curve. Potick (6) measured both the total blood sugar and blood fructose concentrations in the dog following fructose administration; decreasing the carbohydrate content of the diet did not produce a significant alteration in either the fructose or total sugar curves. However, most workers in the field have been concerned with the effect of dietary alterations on glucose tolerance alone, and the phenomenon of starvation diabetes has usually been considered only in terms of altered glucose metabolism.

It has been demonstrated that the metabolic pathways of glucose and fructose are different (7–9) and that administered fructose may be removed from the blood at a normal rate in certain clinical conditions in which glucose tolerance is impaired, such as diabetes mellitus (10), cortisone administration (11), and the stresses of surgery (12), anesthesia (13), and infection (14). These considerations and the results of Potick (6) mentioned above suggested that a similar discrepancy between glucose and fructose metabolism might exist in otherwise normal subjects who were deprived of dietary carbohydrate.

Accordingly, the present studies were designed to compare the effect of dietary carbohydrate deprivation upon intravenous glucose and fructose tolerance in normal human subjects. The results of such a study might yield information regarding the site of the metabolic defect in starvation diabetes.

EXPERIMENTAL PROCEDURES

Six healthy male physicians or medical students, whose ages ranged from 24 to 27 years, were the subjects for these experiments.

Control intravenous glucose tolerance tests were performed on each subject at a time when he had been eating a normal unrestricted diet and had been receiving an oral multivitamin preparation.[3] Four of the subjects then fasted from the evening of one day until the morning of the third subsequent day, when the glucose tolerance test was repeated in a comparable manner. The period of fasting preceding the second test varied from 55 to 60 hours. The intake of water was not restricted and all of the subjects except F.P. received oral multivitamin supplements during this period. Instead of fasting, subjects A.M. and W.C. ate a carbohydrate-free diet consisting of only meat and butter for two days; the glucose tolerance test was repeated on the morning of the third day. Subject A.M. consumed 268 grams of protein and 80 grams of fat each day, while W.C. ate 149 grams of protein and 35 grams of fat daily. They also received oral multivitamin supplements.

At another time control intravenous fructose tolerance tests were performed in the same subjects. After each individual had been subjected to the same form of dietary carbohydrate deprivation, either fasting or a meat diet, for the same period of time as described above, the fructose tolerance test was repeated. In two cases the fruc-

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[2] A preliminary report of this work was presented to the Midwestern Section of the American Federation for Clinical Research, Chicago, Illinois, October 29, 1953.

[3] Avicap® capsules furnished by Burroughs Wellcome & Company and Stuart Formula® tablets supplied by the Stuart Company were used in this study.

tose tolerance tests were performed before the glucose tests.

The hexose tolerance tests were performed in the post-absorptive state with the subject resting in the recumbent position. One gram of glucose or fructose [4] per Kg. of body weight was administered intravenously in one hour. A 10 per cent solution of the hexose in water was used and an infusion pump was employed to insure a uniform rate of administration. Blood samples were obtained through an indwelling needle in an antecubital vein before and at 30 minute intervals after the start of hexose infusion for a total of three hours. The blood samples were analyzed for the following substances by the methods listed:

Glucose or total hexose	Somogyi-Nelson (15)
Fructose	Roe, as modified by Higashi and Peters (16)
Pyruvic acid	Friedemann and Haugen (17)
Citric acid	Natelson, Pincus, and Lugovoy (18) and Taussky and Shorr (19)
Malic acid	Hummel (20)
Inorganic phosphorus	Fiske and Subbarow (21)
Ketone bodies	Eichler and Hindemith (22), modified.

Blood for fructose determination was treated with glucose oxidase before precipitation in order to reduce the concentration of blood glucose to a level at which it did not interfere with the measurement of fructose. In the fructose experiments, the blood glucose level was computed by subtracting the fructose concentration from the total hexose concentration as measured by the Somogyi-Nelson method.

An additional experiment was performed to aid in the assessment of the significance of changes in the concentrations of blood glucose, pyruvic acid, citric acid, malic acid, and plasma inorganic phosphorus. The subjects for this experiment were four male patients whose ages ranged from 52 to 63 years. All four were convalescent from myocardial infarctions which had occurred from 6 to 23 days previously and all were receiving dicumarol anticoagulant therapy. One of these patients had a diabetic-type glucose tolerance curve, but his fasting blood glucose concentration varied from 82 to 89 mg. per 100 ml. With the subject at rest following an overnight fast, peripheral venous blood samples were obtained through an indwelling needle at 30 minute intervals for three hours. No procedure other than the drawing of the blood samples was performed during this period. The samples were analyzed for the substances listed above, and the results are shown in Table I. The mean concentration represents the average of all 28 determinations of a substance in the four patients, and the pooled standard deviation was calculated from the sum of the squared deviations from the mean of each of the four cases. In a similar experiment which has been reported previously (10), the following mean concentrations and pooled standard deviations were obtained in four normal male subjects: glucose, 86 ± 3.3; pyruvic acid, 0.74 ± 0.16; and citric acid, 1.8 ± 0.16 mg. per 100 ml. These results correspond closely to those obtained in the current experiment. When fructose was added to human blood to final concentrations varying between 1.9 and 78 mg. per 100 ml., recoveries within ± 2 per cent of the amount added were obtained by the fructose method described above. In a previous study the mean fasting blood fructose concentration in 17 non-diabetic subjects was found to be 1.7 ± 1.6 mg. per 100 ml. The blood ketone body concentration in normal subjects after an overnight fast is usually less than 1 mg. per 100 ml. by the method used in this study.

The "t" test of Fisher was used to assess the statistical significance of differences between mean values obtained in tests performed after the subjects had been eating a mixed diet and corresponding values after carbohydrate deprivation.

TABLE I

Variations in concentrations of substances in peripheral venous blood specimens obtained at thirty minute intervals for three hours from four subjects in the basal state

Substance	Mean concentration*	Pooled standard deviation
	(*mg. per 100 ml.*)	
Blood glucose	79	±2.9
Blood pyruvic acid	0.86	±0.15
Blood citric acid	2.1	±0.14
Blood malic acid	0.20	±0.017
Plasma inorganic phosphorus	3.3	±0.43

* Average of 28 determinations.

RESULTS

Weight loss

The fasting subjects lost from four to eight pounds during the two day period without food. One of the subjects on the meat diet lost one and one-half pounds, but the weight of the second subject was not altered.

Ketosis

The fasting subjects showed a strongly positive qualitative test for ketone bodies in the urine at the end of the fasting period. In subject R.B., blood ketones were 41 mg. per 100 ml. after the first two day period of fasting and 31 mg. per 100 ml. after his second fast; blood ketones were not measured in the other fasting subjects. After the

[4] The fructose solutions used in this study were supplied by Mead Johnson & Company.

TABLE II

Influence of dietary carbohydrate deprivation on blood glucose and fructose concentrations following intravenous administration of hexoses in normal subjects

Subject	Wt.	Date	Previous diet	Blood glucose (*mg./100 ml.*) Initial concentration	Change from initial concentration: Minutes after start of infusion 30	60	90	120	150	180	Blood fructose (*mg./100 ml.*) Initial concentration	Change from initial concentration: Minutes after start of infusion 30	60	90	120	150	180
	Kg.																
				After glucose (1 Gm./Kg. in 1 hr.)													
F. P.	94	11/21/52	Mixed	98	+193	+302	+150	+ 55	− 2	−26							
R. B.	87	12/ 9/52	Mixed	125	+193	+192	+ 37	− 7	− 48	−64							
J. W.	75	11/18/53	Mixed	95	+162	+224	+ 77	+ 8	− 42	−33							
R. W.	92	12/14/53	Mixed	93	+216	+225	− 27	− 68	− 36	−31							
A. M.	64	12/13/52	Mixed	99	+145	+234	+ 70	− 12	− 42	−27							
W. C.	86	1/10/53	Mixed	91	+171	+247	+ 81	− 8	− 42	−34							
Mean				100	+180	+237	+ 65	− 5	− 35	−36							
F. P.		11/24/52	Fasting	68	+200	+353	+294	+214	+126	+65							
R. B.		12/12/52	Fasting	74	+245	+413	+241	+164	+107	+62							
J. W.		12/ 1/53	Fasting	68	+208	+320	+244	+167	+110	+73							
R. W.		12/17/53	Fasting	71	+217	+300	+152	+ 77	+ 25	+ 4							
A. M.		12/16/52	No CHO	75	+208	+278	+156	+ 58	+ 24	−11							
W. C.		1/14/53	No CHO	68	+206	+330	+252	+174	+119	+25							
Mean				71	+214	+332	+223	+142	+ 85	+36							
				After fructose (1 Gm./Kg. in 1 hr.)													
F. P.		12/ 3/52	Mixed	89	+11	+10	+ 2	+ 2	−10	+ 1	7	+72	+81	+14	+ 3	0	−3
R. B.		12/27/52	Mixed	100	−22	− 1	−13	− 8	−15	−10	<1	+64	+78	+28	+12	+7	+4
J. W.		12/15/53	Mixed	78	+ 1	+34	− 3	−24	−19	− 6	1	+73	+76	+24	+ 9	+3	+4
R. W.		1/26/54	Mixed	86	+19	+33	−16	−40	−30	−14	3	+96	+99	+38	+10	+4	+2
A. M.		11/28/52	Mixed	75	−11	+ 8	−18	−15	−12	− 8	2	+86	+94	+28	+13	+8	+3
W. C.		12/17/52	Mixed	66	+14	+53	+21	− 5	+ 2	− 2	2	+89	+88	+31	+13	+3	+3
Mean				82	+ 2	+23	− 4	−15	−14	− 6	3	+80	+86	+27	+10	+4	+2
F. P.		12/ 6/52	Fasting	72	+17	+29	+22	+24	+25	+17	<1	+90	+86	+25	+12	+4	+3
R. B.		12/31/52	Fasting	60	+34	+46	+45	+27	+19	+23	3	+98	+83	+27	+12	+4	+2
J. W.		12/18/53	Fasting	68	+ 8	+38	+32	+18	+ 6	+ 2	1	+59	+76	+27	+10	+5	+2
R. W.		1/29/54	Fasting	76	+ 3	+26	+23	+ 9	+ 9	− 1	2	+73	+87	+26	+14	+4	+3
A. M.		12/ 1/52	No CHO	70	+44	+38	+35	+29	+ 5	+ 9	2	+71	+86	+31	+12	+6	+2
W. C.		12/20/52	No CHO	58	+24	+41	+24	+18	+16	+12	3	+95	+78	+27	+10	+4	+3
Mean				67	+21	+36	+30	+21	+13	+10	2	+81	+83	+27	+12	+4	+2

meat diet, one subject had a blood ketone level of 8 mg., and the other had a concentration of 26 mg. per 100 ml. on one occasion and 10 mg. per 100 ml. after the second dietary period.

Concentrations of metabolites in blood or plasma in postabsorptive state

These data are included in Tables II, III, and IV. In every instance carbohydrate deprivation for two days resulted in a decrease in the fasting blood glucose concentration; the average control value plus or minus one standard deviation was 91 ± 15 mg. per 100 ml., as compared to 69 ± 6 mg. per 100 ml. after dietary restriction ($p < 0.01$). In 9 out of 12 instances, the fasting blood pyruvic acid concentration was higher after carbohydrate deprivation; the average control value was 1.0 ± 0.2 mg. per 100 ml. and the average after dietary restriction was 1.2 ± 0.2 mg. per 100 ml. ($p < 0.05$). Similarly, the average fasting plasma citric acid level was higher (2.2 ± 0.3 mg. per 100 ml.) after dietary restriction than before (1.8 ± 0.4 mg. per 100 ml.); such a difference was seen in 10 of 11 instances, $p < 0.02$. Reduction of the quantity of dietary carbohydrate did not produce a consistent alteration in the fasting concentration of blood malic acid or plasma inorganic phosphorus.

Since the results of the hexose tolerance tests in the subjects who fasted were similar to those obtained in subjects on the meat diet, they will not be described separately but will be considered as one group. The data are contained in Tables II, III, and IV, and the tolerance curves are shown in Figure 1.

Glucose tolerance tests

During the control tests the blood glucose concentration rose to an average of 237 ± 36 mg. per 100 ml. above the initial level and returned to or below the initial level in 120 minutes, except in the cases of F.P. and J.W., in which the initial concentration was not attained until 150 minutes. In the tests performed in the same subjects after two days of dietary carbohydrate deprivation, the maximum glucose rise exceeded ($p < 0.01$) that of the control test in all six cases. The maximum rises exceeded those of the control tests by 44 to 221 mg. per 100 ml. and the average maximum rise above the initial level was 332 ± 47 mg. per 100 ml. From 60 through 180 minutes the blood glucose concentrations were consistently higher than in the control tests, and in only two cases had the blood glucose concentration returned to the initial level in 180 minutes.

During the control tests all the subjects showed a rise in blood pyruvic acid concentration which varied from 0.2 to 1.5 mg. per 100 ml. and averaged 0.5 ± 0.5 mg. per 100 ml. After dietary restriction the average increase in blood pyruvic acid was only 0.2 ± 0.2 mg. per 100 ml. with a variation from 0 to 0.6 mg. per 100 ml. The difference between the mean blood pyruvic acid rises before and after dietary carbohydrate restriction did not prove to be statistically significant ($p > 0.2$).

When glucose was given, the concentration of the blood citric acid decreased slightly in five of the six subjects. Carbohydrate deprivation did not alter this response significantly; when the mean changes in blood citric acid concentration

TABLE III

Influence of carbohydrate deprivation on blood pyruvic acid and citric acid concentrations following intravenous administration of hexoses in normal subjects

Subject	Previous diet	Blood pyruvic acid (*mg./100 ml.*)							Blood citric acid (*mg./100 ml.*)						
		Initial concentration	Change from initial concentration, minutes after start of infusion						Initial concentration	Change from initial concentration, minutes after start of infusion					
			30	60	90	120	150	180		30	60	90	120	150	180
After glucose (1 Gm./Kg. in 1 hr.)															
F. P.	Mixed	0.8	0	+0.2	+0.6	+0.5		+0.1	1.6	+0.3	+0.2	+0.2	+0.1		−0.1
R. B.	Mixed	0.9	+1.1	+1.5	+1.0	+0.3	0	−0.2	2.2	0	−0.1	−0.1	−0.1	−0.5	−0.5
J. W.	Mixed	1.1	+0.2	+0.1	+0.1	−0.1	−0.2	+0.1	2.6	−0.2	−0.2	0			
R. W.	Mixed	0.9	−0.4	+0.4	−0.3	0	+0.3	+0.5	2.0	−0.2	−0.1	0		−1.0	−0.1
A. M.	Mixed	0.9	−0.1	+0.3	+0.3	0	−0.1	−0.3	2.1	−0.1	−0.1	−0.1	−0.1		−0.5
W. C.	Mixed	1.2	+0.2	0	+0.2	+0.1	−0.5	−0.5	1.5	−0.1	0	+0.2	0	−0.2	−0.2
Mean		1.0	+0.2	+0.4	+0.3	+0.1	−0.1	−0.1	2.0	0	−0.1	0	0	−0.6	−0.3
F. P.	Fasting	1.2	+0.4	+0.3	+0.1	0	+0.2	+0.4	2.0	+0.1	+0.2		0	+0.2	−0.1
R. B.	Fasting	1.2	−0.1	+0.1	+0.2	0	0	−0.3	2.6	0	−0.2	+0.2	−0.2	−0.2	−0.4
J. W.	Fasting	1.2	+0.1	+0.2	−0.4	+0.5	+0.5	+0.6	2.8	−0.4	−0.3	−0.4	−0.4	−0.7	−0.3
R. W.	Fasting	1.3	−0.2	−0.1	0	+0.3	+0.4	0	2.6	−0.3	−0.2	−0.2	−0.1	−0.5	−0.1
A. M.	No CHO	1.5	−0.2	0	0	−0.1	0	−0.3	2.1	−0.1	−0.4	−0.2	−0.6	−0.6	−0.7
W. C.	No CHO	0.9	+0.6	+0.4	+0.3	+0.3	+0.3		1.8	−0.1	−0.1	−0.5	−0.5	−0.6	−0.5
Mean		1.2	+0.1	+0.2	0	+0.2	+0.2	+0.1	2.3	−0.1	−0.2	−0.2	−0.3	−0.4	−0.4
After fructose (1 Gm./Kg. in 1 hr.)															
F. P.	Mixed	1.0	+1.8	+2.6	+0.9	+0.3	+0.6	+0.4	1.4	+0.2	+0.5	+0.4	+0.2		−0.2
R. B.	Mixed	1.5	−0.1	+1.3	+0.5	+0.5	+0.3	−0.2	1.6	+1.0	+0.6	+0.8	+0.5	+0.4	+0.4
J. W.	Mixed	0.9	+0.9	+1.3	+0.5	+0.1	−0.1	−0.1							
R. W.	Mixed	0.8	+1.2	+1.1	+0.4	−0.1	−0.2	−0.2	2.0	+0.1	+0.1	+0.4	0	−0.2	−0.4
A. M.	Mixed	1.0	+1.1	+0.9	+0.1	+0.4	+0.4	−0.1	1.6	+0.2	+0.3	+0.4	+0.3		−0.2
W. C.	Mixed	1.4	+1.0	+1.3	+0.5	−0.4	−0.8	−0.3	1.6	+0.1	+0.4	+0.3	+0.2	−0.3	−0.3
Mean		1.1	+1.0	+1.4	+0.5	+0.1	0	−0.1	1.6	+0.3	+0.4	+0.5	+0.2	0	−0.1
F. P.	Fasting	1.2	+0.5	+1.7	+0.7	+0.2	0	−0.1	1.8	0	−0.2	+0.1	+0.4		0
R. B.	Fasting	1.1	+1.7	+2.7	+1.9	+0.7	+0.4	+0.1	2.1	0	+0.5	+0.4	+0.3	−0.2	+0.1
J. W.	Fasting	1.4	+0.5	+2.0	+1.0	+1.1	+0.4	+0.1							
R. W.	Fasting	1.2	+0.7	+2.1	+1.8	+0.6	+0.4	+0.1	2.5	+0.2		+0.4	+0.6	+0.3	+0.1
A. M.	No CHO	1.3	+1.0	+1.6	+0.3	0	+0.2	−0.5	2.3	+0.1	−0.1	−0.1	−0.2		−0.6
W. C.	No CHO	1.2	+1.2	+1.7	+1.1	+0.2	−0.1	−0.4	2.1	0	+0.2	+0.2	+0.2	−0.2	−0.4
Mean		1.2	+0.9	+2.0	+1.1	+0.5	+0.2	−0.1	2.2	+0.1	+0.1	+0.2	+0.3	0	−0.2

TABLE IV

Influence of carbohydrate deprivation on blood malic acid and plasma inorganic phosphorus concentrations following intravenous administration of hexoses in normal subjects

Subject	Previous diet	Blood malic acid (*mg./100 ml.*)							Plasma inorganic phosphorus (*mg./100 ml.*)						
		Initial concentration	Change from initial concentration, Minutes after start of infusion						Initial concentration	Change from initial concentration, Minutes after start of infusion					
			30	60	90	120	150	180		30	60	90	120	150	180
After glucose (1 Gm./Kg. in 1 hr.)															
F. P.	Mixed	0.15	+0.01	−0.01	+0.02	+0.02		−0.01	3.6	−0.4	−0.8	−1.1	−1.1		−1.6
R. B.	Mixed	0.17	+0.02	−0.01	−0.03	0	+0.01	−0.02	4.0	−0.7	−0.2	−1.8	−2.4	−1.9	−2.0
J. W.	Mixed								3.8	−0.6	−1.2	−1.4	−1.4	−1.3	−1.6
R. W.	Mixed								4.0	−0.6	−0.7	−0.9	−0.8	−1.4	−0.9
A. M.	Mixed	0.14	+0.04	+0.04	+0.02	0		0	4.5	−0.6	−0.8	−1.4	−1.3	−1.3	−1.1
W. C.	Mixed	0.13	+0.03	+0.02	+0.04	+0.03	+0.01	+0.02	4.2	−0.4	−0.9	−1.4	−1.6	−1.3	−1.3
Mean		0.15	+0.02	+0.01	+0.01	+0.01	+0.01	0	4.0	−0.6	−1.1	−1.3	−1.4	−1.4	−1.4
F. P.	Fasting	0.17	0	0		0	+0.02	0	4.1	−0.3	−0.9		−1.3	−1.7	−1.7
R. B.	Fasting	0.15	0	0	+0.04	0	−0.01	+0.03	3.2	−0.7	−1.3	−1.5	−1.4	−0.9	−1.4
J. W.	Fasting								3.8	−0.6	−0.8	−1.3	−1.1	−1.4	−1.3
R. W.	Fasting								4.5	−0.6	−1.3	−1.7	−1.3	−1.0	−0.7
A. M.	No CHO	0.16	+0.03	+0.02	+0.05	+0.04	+0.04	+0.06	3.8	−1.0	−1.4	−2.0	−1.6	−1.7	−1.5
W. C.	No CHO	0.17	−0.01	+0.02	−0.01	+0.01	+0.02	+0.03	3.4	−0.1	−0.6	−1.0	−1.0	−1.5	−1.4
Mean		0.16	+0.01	+0.01	+0.02	+0.01	+0.02	+0.03	3.8	−0.6	−1.0	−1.5	−1.3	−1.4	−1.3
After fructose (1 Gm./Kg. in 1 hr.)															
F. P.	Mixed	0.21	+0.01	0	−0.02	−0.06		−0.06	4.5	−0.8	−0.7	−1.1	−1.1		−1.3
R. B.	Mixed	0.17	+0.04	+0.06	+0.02	−0.02	0	−0.01	4.0	−0.6	−0.6	−0.7	−0.7	−1.0	−1.1
J. W.	Mixed								3.8	−1.1	−0.9	−0.4	−0.6		−0.5
R. W.	Mixed								5.0	−1.7	−1.2	−0.9	−1.2	−1.1	−0.7
A. M.	Mixed	0.14	+0.01	+0.04	+0.01	+0.02		−0.01	3.7	−0.5	−0.4	−0.4	−0.9		−0.5
W. C.	Mixed	0.16	+0.04	+0.04	+0.04	+0.01	−0.01	+0.03	4.4	−0.7	−1.1	−0.4	−0.9	−1.2	−1.0
Mean		0.17	+0.02	+0.04	+0.01	−0.02	0	−0.01	4.2	−0.9	−0.8	−0.6	−0.9	−1.1	−0.8
F. P.	Fasting	0.14	+0.01	+0.03	+0.02	+0.01		0	4.3	−0.8	−1.0	−0.6	+0.1		−1.0
R. B.	Fasting	0.11	+0.07	+0.08	+0.07	+0.06	+0.04	+0.04	2.7	−0.5	−0.5	−0.1	+0.2	−0.2	−0.2
J. W.	Fasting								3.9	−0.6	−0.8	−0.6	−0.7	−0.7	−1.0
R. W.	Fasting								4.2	−0.5	−0.6	−0.4	−0.4	−0.4	−0.3
A. M.	No CHO	0.17	0	+0.05	−0.02	+0.01		+0.01	3.7	−0.2	−0.6	−0.5	−1.1		−0.8
W. C.	No CHO	0.15	+0.03	+0.07	+0.02	+0.01	−0.01	−0.02	3.7	−0.7	−0.9	−0.6	−0.5	−0.8	−0.6
Mean		0.14	+0.03	+0.06	+0.02	+0.02	+0.01	+0.01	3.8	−0.6	−0.7	−0.5	−0.4	−0.5	−0.6

were compared to the mean changes at corresponding time intervals in the control tests, p was consistently greater than 0.05. Glucose administration was associated with minimal increases in the blood malic acid concentration both before and after carbohydrate deprivation. In every instance glucose produced a fall in the level of plasma inorganic phosphorus; the average maximum decrease was 1.7 ± 0.4 mg. per 100 ml. in the control tests and 1.6 ± 0.2 mg. per 100 ml. after dietary carbohydrate restriction ($p > 0.5$).

Fructose tolerance tests

In the control tests the blood fructose concentration reached an average maximum value of 89 ± 10 mg. per 100 ml. and returned to the initial level by 150 or 180 minutes. In tests performed after two days of carbohydrate deprivation the fructose concentration reached an average maximum value of 91 ± 8 mg. per 100 ml. ($p > 0.5$). In one case the rise was 20 mg. per 100 ml. greater than in the control test, and in another case it was 12 mg. per 100 ml. less; in the remaining cases the maximum values attained in the two tests varied by less than 10 mg. per 100 ml. The rate of decline of the blood fructose concentration was similar to that observed in the control tests.

In three of the six cases, the blood glucose rise associated with fructose administration was greater after carbohydrate restriction than in the well-fed state, in one case it was similar, and in two cases it was less after carbohydrate deprivation. In all instances the return of the blood glu-

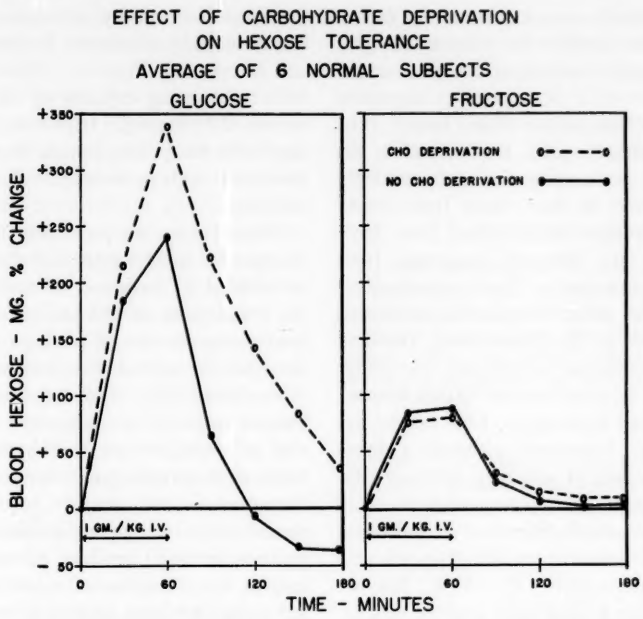

FIG. 1. AVERAGE INTRAVENOUS GLUCOSE AND FRUCTOSE TOLERANCE CURVES OF SIX NORMAL SUBJECTS FOLLOWING A NORMAL DIET (SOLID LINES) AND FOLLOWING TWO DAYS OF FASTING OR A CARBOHYDRATE-FREE DIET (INTERRUPTED LINES)

Blood hexose concentrations are plotted in terms of variation from the fasting level.

cose to the initial level was delayed after dietary restriction. The mean blood glucose changes were significantly different ($p < 0.01$) in the control and postrestriction tests at 90, 120, 150, and 180 minutes.

Five of the six subjects showed a greater increase in blood pyruvic acid concentration after carbohydrate deprivation. The average maximum rise was 1.4 ± 0.6 mg. per 100 ml. in the control test and 2.0 ± 0.4 per 100 ml. after dietary restriction ($0.05 < p < 0.1$).

During the fructose tolerance tests there was a small increase in the concentration of blood citric acid in each instance in contrast to the decrease produced by glucose administration; the magnitude of the increase was not altered in a consistent manner by dietary restriction, although the mean rise at 90 minutes was less ($0.05 > p > 0.02$). There were small increases in the blood malic acid level during the tests performed both before and after carbohydrate deprivation; however, the mean changes from the initial level were not significantly different at any point in the control and postdeprivation tests. Fructose administration was accompanied by a fall in the concentration of plasma inorganic phosphorus in each instance. The average maximum phosphorus decrease was 1.2 ± 0.3 mg. per 100 ml. in the control tests and 0.8 ± 0.2 mg. per 100 ml. after dietary restriction ($0.02 > p > 0.01$).

DISCUSSION

The results of these studies confirm previous observations that dietary carbohydrate deprivation decreases the rate of removal of administered glucose from the blood. Under the same circumstances, the rate of removal of administered fructose is not measurably altered. These findings afford further evidence that in man the predominant pathway for glucose metabolism differs from the pathway for fructose metabolism. They sug-

gest that the metabolic defect induced by carbohydrate deprivation involves the pathway for glucose utilization without impairing fructose metabolism. Certain metabolic derangements have been demonstrated in liver slices from fasted rats. Wyshak and Chaikoff (23) reported that the conversion of glucose to CO_2, glycogen, and fatty acids was depressed in liver slices from fasted rats; CO_2 and glycogen were formed from fructose at a normal rate, although lipogenesis from this hexose was impaired. These investigators suggested that the defect in glucose utilization probably occurred at the glucokinase reaction, while the phosphorylation of fructose was under the influence of a separate enzyme (fructokinase) and was not altered by fasting. Our results are consistent with this hypothesis, although a defect in glucose metabolism at any step between glucose transport and the triose phosphate level could account for the observed difference in the effect of carbohydrate deprivation on the disappearance of glucose and fructose from the blood. Kuyper (24) found that the fructokinase activity was diminished in liver slices from fasted rats; however, our studies do not indicate a significant alteration in the overall rate of fructose removal such as might be observed if the phosphorylation of fructose were impaired by carbohydrate deprivation in man.

The fact that results similar to those of the present study were obtained when glucose and fructose were administered to diabetic subjects (10) suggests the possibility that a lack of insulin effect may be involved in the changes induced by carbohydrate deprivation. The available data regarding the role of insulin in starvation diabetes are conflicting. A diminished insulin effect might be due to a decreased supply of endogenous insulin or to the presence of insulin antagonists. Best, Haist, and Ridout (25) observed a decrease in the insulin content of the pancreas of fasted rats. However, Bergman and Drury (26) found that rabbits which had been fasted prior to evisceration exhibited a diminished rate of glucose utilization in comparison to non-fasted eviscerated rabbits; since both groups of animals were depancreatized, this phenomenon apparently did not depend upon differences in the amount of available insulin at the time that glucose utilization was measured. Himsworth (27) reported that the insulin sensitivity of normal human subjects was reduced by a decrease in dietary carbohydrate, and Mirsky and Perisutti (28) found that the insulin-inactivating capacity of rat liver slices was increased by fasting. However, the importance of insulin in the production of the metabolic defects associated with carbohydrate deprivation remains unsettled.

There is also the possibility that the metabolic changes associated with carbohydrate deprivation are related to increased adrenal cortical activity. An impairment of glucose tolerance with no alteration in the rate of fructose removal has been described in normal men following a single dose of cortisone (11). Engel's observation (29) that glucose tolerance is unimpaired by a 48 hour period of fasting or low carbohydrate intake in patients with adrenal insufficiency also suggests that the adrenal cortex may be involved. In experimental animals, complete inanition (30) or a high protein diet (31) produce adrenal cortical hypertrophy, but the effect of a low carbohydrate diet *per se* has not been studied adequately. In Cushing's syndrome (32–34) and in patients receiving adrenocorticotropic hormone (ACTH) or adrenal cortical steroids (33–36) the concentration of blood pyruvic acid is frequently elevated in the postabsorptive state. It is of interest in this regard that in 9 of our 12 paired experiments the concentration of blood pyruvic acid in the postabsorptive state was higher after carbohydrate deprivation than in the control test. In Cushing's syndrome an abnormally high rise in blood pyruvic acid has been observed after fructose infusion (37); fructose administration produced a greater rise in the blood pyruvic acid concentration after carbohydrate deprivation than in the control test in five of our six subjects. Abnormally great and prolonged elevations of the blood pyruvic acid concentration have been reported after glucose administration in Cushing's syndrome (32, 33), and the same phenomenon has been observed in patients receiving ACTH or adrenal cortical steroids (33, 34). However, in the present experiments such a pyruvic acid change was recorded in only one of six subjects when glucose was administered after carbohydrate deprivation. Because more direct measurements of adrenal cortical function in man during a relatively brief period of carbohydrate deprivation

are not available, the relationship of the adrenal cortex to the observed metabolic changes cannot be evaluated accurately.

Engel (29) has reported that the glucose tolerance test is not altered by fasting in the patient with pituitary insufficiency, and Himsworth and Scott (38) found that glucose tolerance was not impaired by a low carbohydrate diet in the hypophysectomized rabbit. Recant (39) investigated a respiratory quotient (R.Q.) depressing factor of anterior pituitary origin. This substance inhibited the rise in R.Q. which occurred when glucose or hexose diphosphate were added to a rat diaphragm preparation but did not inhibit the R.Q. rise produced by 3-phosphoglyceric acid. A similar R.Q. depressing factor was demonstrated in the serum of fasted normal rats but was absent from the serum of fasted hypophysectomized rats and normal rats on an adequate diet. Although these observations suggest that the anterior pituitary may be involved in the production of the metabolic changes associated with dietary carbohydrate deprivation, the results of other investigators are not in agreement. For example, two groups of workers (40, 41) have altered the glucose tolerance curves of hypophysectomized dogs by reducing the carbohydrate content of the diet. As in the case of the other endocrine glands, sufficient information is not available to assess the role of the anterior pituitary in the production of the metabolic changes of starvation diabetes.

From a teleological viewpoint, it is of interest that the metabolism of the most important carbohydrate fuel of the body, glucose, is impaired in so many conditions, including starvation, diabetes mellitus, and stress; while the utilization of fructose, which apparently plays a relatively minor role quantitatively in the normal body economy, proceeds at an unchanged rate under the same circumstances. In starvation, the net impairment of glucose utilization may represent a mechanism for the maintenance of an adequate supply of glucose for the central nervous system which is dependent upon this particular hexose.

SUMMARY

1. Intravenous glucose and fructose tolerance tests were performed in six normal subjects before and after two days of dietary carbohydrate deprivation.

2. The rate of removal of administered glucose from the blood was diminished after carbohydrate deprivation, while fructose disappeared at a normal rate.

3. These results afford further evidence that the metabolism of fructose differs from the metabolism of glucose in man.

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CONTENT AND COMPOSITION OF THE MUCOPROTEIN FRACTION OF HUMAN SERUM (SEROMUCOID) IN DISEASE, WITH SPECIAL REFERENCE TO HEMATOLOGIC DISORDERS [1]

By ELLY MOSCHIDES,[2] MARIO STEFANINI,[3] SERGIO I. MAGALINI,[4] AND STEN A. KISTNER [5] WITH THE TECHNICAL ASSISTANCE OF ROSE MELE

(From the Joseph Stanton Memorial Laboratories, St. Elizabeth's Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.)

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Significant alterations of content, distribution, electrophoretic mobility and, possibly, composition of carbohydrate-containing proteins in many diseases have been described by both chemical and electrophoretic methods. From a summary of the pertinent observations in the literature (Table I), it may be concluded that the carbohydrate content of proteins is high in many disease states; also, that the electrophoretic distribution of polysaccharides or their chemical precipitation with the various protein fractions may often be abnormal. Thus, the study of carbohydrate-containing proteins of serum is a potentially important and significant tool in the detection of basic molecular alterations, which may be significant in the understanding of the pathogenesis of disease.

We have attempted in a number of disease states a systematic study of the content and in the chemical composition of one type of carbohydrate-containing proteins, the mucoproteins. Because of the major interest of our Institution and our previous work on the subject particular emphasis was directed to hematologic disorders. The serum mucoprotein fraction was selected for this study because: a) it shows significant and wide variations in disease (Table I); b) fairly reliable techniques are available for its isolation and for the analysis of some of its constituents. Total mucoproteins, mucoprotein-bound hexoses and hexosamine were studied. For comparison, values for total protein, total hexosamine and total non-glucosamine protein-bound hexoses were also obtained for all sera. Our work was handicapped by the present confusion in classification and definition of the polysaccharide-containing proteins. These have been divided by Meyer (1) into glycoproteins and mucoproteins. The two types appear similarly constituted with a mucopolysaccharide held to a protein moiety by a firm chemical bond. However, in mucoproteins, the degree of alcohol solubility is higher and the hexosamine content is more than 4 per cent weight. In glycoproteins, hexosamine represents less than 4 per cent. In this study the definition of mucoprotein, as suggested by Winzler, Devor, Mehl, and Smyth (2), was applied to that fraction of serum proteins soluble in perchloric acid solution, and precipitable by phosphotungstic acid. The fraction we studied appeared comparable to the seromucoid of Rimington (3) and the seroglycoid of Hewitt (4). Since we considered as mucoproteins a group of proteins of serum on criteria of solubility alone, only relative conclusions were possible. In particular, our findings have no bearing on the exact nature of mucoproteins and on their relationship to other carbohydrate-containing proteins.

METHODS

Total serum protein (T.P.) was determined by the method of Gornall, Bardawill, and David (5), which employs the biuret reaction; total non-glucosamine, protein-bound hexoses (T.Ph.) by the procedure of Sheppard and Everett (6); total hexosamine (T.Ha) by the method of Rimington (3); (d) serum mucoproteins (T.Mp.) were separated as described by Winzler and co-workers (2). This method is based on the precipita-

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[2] Research Assistant, Joseph Stanton Memorial Laboratories.

[3] Established Investigator, American Heart Association.

[4] Formerly Damon Runyon Cancer Research Fellow. Present address: Cancer Research Foundation, Boston.

[5] Fellow of the American-Scandinavian Foundation. Present address: Department of Biochemistry, Nobel Institute, Stockholm, Sweden.

TABLE I

*Serum total hexoses, protein-bound hexoses, total hexosamine and mucoprotein in disease: A summary**

Total hexoses	*Elevated* in advanced tuberculosis (21, 22, 16), sarcoidosis and cancer (22, 16), rheumatic fever in relapse (9), rheumatoid arthritis (15), diabetes mellitus with complications (14), disseminated malignancies (23, 24), leukemia, lymphoma, chronic infections, colitis, acute glomerulonephritis, prostatic hypertrophy (24).
Protein-bound hexoses	*Elevated* in carcinoma, benign tumors, arthritis, bacterial infections (17), disseminated malignancies (25, 26), tuberculosis (27, 28). *Hexoses in serum albumin fraction:* elevated in normal pregnancy (29), more so in carcinoma (17) and rheumatic fever (9). In multiple myeloma, the pathological protein contains maximum amount of polysaccharides (30). In pneumonia, polysaccharides are high in all electrophoretic fractions (31).
Protein-bound hexoses: total protein ratio	*Elevated* in rheumatoid arthritis, in relation to severity of symptoms (32).
Total hexosamine	*Elevated* in rheumatic fever (9), pneumonia (33), carcinoma. Hodgkin's disease, leukemias, rheumatoid arthritis, periarteritis nodosa, rheumatic fever, chronic infection, pneumonia, myocardial infarction (34), lupus erythematosus (35), gout, cirrhosis of the liver, hepatitis (18). Hexosamine is highest in the gamma globulin fraction of patients with lupus erythematosus (36). In other diseases, the α_1-globulin contains most of the increased hexosamine (18).
Mucoprotein (seromucoid)	*Elevated* in diabetes mellitus with complications (14), myocardial infarction (37), carcinoma and pneumonia (19), rheumatic fever (38), trauma (12). *Decreased* in liver disease, some endocrine dysfunctions, nephrosis (12).
Mucoprotein-bound hexoses	*Elevated* in rheumatoid arthritis (15).

* The terminology used in this table is in agreement with that used in the text.

tion of all proteins by perchloric acid and subsequent isolation of mucoproteins which are insoluble in phosphotungstic acid.[6] After isolation, the following components of mucoproteins were determined: a) protein moiety (T.Mp.); b) hexoses (Mh.); c) hexosamine (Mha.). Mean values and standard deviation were calculated for each value according to the formula:

$$\sigma = \sqrt{\frac{\Sigma i(\bar{x} - xi)^2}{n}},$$

where $\bar{x}$ represented the mean, xi the i^{th} individual determination, and n the number of determinations. The significance of our results was further evaluated by means of the formula (7):

$$t = \frac{\overline{X}_1 - \overline{X}_2}{S_{1-2}} \sqrt{\frac{N_1 \cdot N_2}{n_1 + n_2}}$$

where S_{1-2} equalled

$$\sqrt{\frac{\Sigma x_1^2 + \Sigma x_2^2}{N_1 + N_2 - 2}}.$$

In the latter formula, $\overline{X}_1$ indicated the mean of normal values; $\overline{X}_2$, the mean of the values being investigated; N_1, the number of samples in the normal group; N_2, the number of samples in the group investigated; x_1 the square root of the differences of each sample in the normal group from the mean value; x_2 the same value for the group investigated. The significance of t values was judged on the basis of a standard table (8).

Materials (Table IV)

Eight healthy individuals and 111 patients were included in this study. Eighty-eight patients represented cases seen in the wards of our Institution. Serum for an additional twenty-three patients was obtained through the courtesy of Drs. Jean Dausset (Centre National des Transfusions Sanguines, Paris, France), James Monto (Henry Ford Hospital, Detroit, Michigan) and Donat Cyr (Lahey Clinic, Boston, Massachusetts). These samples may be recognized in all tables since they are indicated by number, rather than by patient's initials; they were included in the series to verify the reliability of results against possible errors in diagnosis. Serum from local patients was promptly frozen and stored at $-20°$ C. until used. Sera from other areas were sent by air in containers filled with dry ice. In each in-

[6] Winzler and co-workers (2) have indicated that dilution of serum and final molarity of perchloric acid are essential to optimal precipitation of a constant amount of mucoprotein. They have advised a concentration of 0.6 M perchloric acid. Results indicated in Tables II and III show that dilution and molarity of perchloric acid were of relatively little bearing on the final yield of mucoproteins. Thus, a molarity of 0.24 M was used in the course of this study, a figure which seemed to assure the complete precipitation of the total protein fraction with a minimum amount of reagent. The serum was diluted 1:5 with perchloric reagent as suggested by Winzler.

TABLE II

Effect of the dilution of serum on the recovery of mucoproteins and their composition: A representative experiment

		Weight of dry precipitate	Composition of mucoproteins		
			Protein	Hexoses	Hexosamine
		mg. %	*mg. %*	*mg. %*	*mg. %*
(a) Normal serum dilution	1:1	83	80	4.2	9.6
	1:2	83	80.9	4.21	9.7
	1:5	82	82	4.29	9.77
(b) Abnormal serum dilution (lymphosarcoma: S. R., ♀, 38)	1:1	296	296	34	15.5
	1:2	295.3	295.5	33.4	15.25
	1:5	296	296	33.6	15.4

TABLE III

Total mucoprotein (T.Mp.), mucoprotein-bound hexoses (Mh.) and hexosamine (Mha.) from serum treated with decreasing concentration of perchloric acid *

Molarities of perchloric acid	0.6			0.45			0.3			0.225			0.15		
	T.Mp.	Mh.	Mha.	T.Mp.	Mh.	Mha.	T.Mp.	Mh.	Mha.	T.Mp.	Mh.	Mha.	T.Mp.	Mh.	Mha.
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
Normal serum	98.8	6.2	11.5	98.6	6.2	11.5	98.8	6.1	11.6	98.8	6.1	11.5	98	5.75	11
	100	6.3	11.7	101	5.2	11.7	100	6.1	11.5	98	6.2	11.6	92	5.6	10.9
Pathological serum	190	12.6	15.1	190	12.5	15	190	12.6	15	189	12.6	15	162	12	14.5
	218	13.0	19.1	217	12.9	19.0	218	12.9	19.1	218	13.0	19.2	209	11.9	17.3

* Averages of duplicate experiments on each serum.

stance, diagnosis was established by the usual hematologic criteria, and proven by either biopsy of involved tissues or autopsy findings. Since many diseases are accompanied by major alterations in content of serum glycoproteins and mucoproteins, cases selected for the present study, as far as could be determined, were free from infections, thrombosis, or hepatic and renal disorders. No definitive data appeared available to indicate whether treatment (myelo-suppressive drugs, X-ray radiation, and so on) may influence the level and composition of mucoproteins.[7] In our experience, the amount and the composition of the serum mucoprotein fraction were more closely related to the state of remission or relapse, than to the effects of therapy. Usually, therefore, blood was collected from patients in clinical relapse, who were untreated at the time of blood collection.

RESULTS

The conclusions to follow are based primarily on the results of the t test as a criterion of the statistical significance of our findings.

[7] Steroids, chemotherapeutic agents of various nature, X-ray radiation, and so on might considerably modify the content and composition of mucoproteins in disease. The elevated content of mucopolysaccharide in the serum of patients with rheumatic fever decreases significantly following steroid therapy (9). In guinea pigs (10) and in rats (11), however, no correlation has been found between administration of steroids and variations in protein-bound hexoses.

A. Normal sera (Table V)

Our findings agreed closely with those reported in the literature. They represented base values with which abnormal findings were compared.

TABLE IV

Sera studied in the course of these observations

Iron deficiency anemia	4	
Pernicious anemia	6	
Polycythemia vera	4	
Myeloid metaplasia	4	
Congenital spherocytosis	4	
Acute leukemia: myelogenous	5	
histiocytic	3	11
erythroblastic	1	
unclassified	2	
Chronic leukemia: myelogenous	7	16
lymphocytic	9	
Hodgkin's disease: granuloma	5	10
sarcoma	5	
Lymphoblastoma	3	
Lymphosarcoma	9	
Reticulum cell sarcoma	8	
Mycosis fungoides	5	
Multiple myeloma	6	
Acquired auto-immune hemolytic anemia	3	
Idiopathic thrombocytopenic purpura	5	
Lupus erythematosis disseminatus	5	
Disseminated malignancies	8	
		111
Normal sera	8	
Total		119

TABLE V

Serum protein-bound hexoses in normal subjects *

Subject	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
S. M., ♂, 28	7,600	100	100	95	5.355	10.20
M. M., ♀, 28	6,800	95	85	90	4.165	10.20
E. M., ♀, 29	7,000	125	107	105	8.500	12.75
W. B., ♂, 24	7,000	99	88	115	10.200	12.75
R. M., ♀, 32	7,200	120	100	85	4.250	9.35
N. M., ♀, 25	7,700	115	105	100	7.140	11.90
M. S., ♂, 38	7,500	107	98	90	4.505	9.35
S. K., ♂, 29	7,900	115	106	110	7.650	13.60
Average	7,337.5	109.5	98.6	98.8	6.471	11.26
σ	367.21	10.17	7.647	9.92	2.098	1.58

* As in each table, analytical values and standard deviation (σ) are given for the following: Total protein (T.P.), protein-bound hexoses (T.Ph.), total hexosamine (T.Ha), total mucoprotein (T.Mp.), mucoprotein-bound hexoses (Mh.), and mucoprotein-bound hexosamine (Mha).

TABLE VI

Serum protein-bound hexoses in acute leukemia

Type	Case	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
		mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
Unclassified	No. 3005	7,300	138	128	186	8.84	13.60
Granulocytic	No. 11	7,600	130	115	246	27.63	23.80
Granulocytic	No. 12	7,800	135	120	171	24.40	21.25
Granulocytic	No. 13	7,600	160	150	228	12.50	12.75
Histiocytic	D. M., ♀, 38	7,200	147	140	310	42.50	12.75
Granulocytic	L. S., ♀, 76	7,200	147	120	253	38.25	10.20
Histiocytic	C. P., ♂, 29	7,300	150	120	103	15.30	13.60
Erythroblastic	P. McG., ♂, 52	7,000	160	140	123	20.91	18.70
Lymphocytic	J. T., ♂, 5	6,100	190	120	123	8.67	10.20
Granulocytic	C. V., ♀, 54	7,000	190	140	140	16.58	15.30
Granulocytic	R. Q., ♂, 57	6,100	185	110	117	9.69	11.90
Average		7,109.1	157.5	127.5	181.8	20.48	14.91
σ		531.64	19.88	12.3	65.34	11.14	4.26
t		0.989	5.63	4.95	3.28	4.38	2.15

TABLE VII

Serum protein-bound hexoses in chronic leukemia (myelogenous and lymphocytic)

Case	Type	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
		mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
G. D., ♀, 53	Granulocytic	5,900	125	110	113	12.50	13.60
J. O., ♂, 29	Granulocytic	6,900	130	115	118.6	15.30	17.00
No. 7	Granulocytic	6,000	85	120	105.2	15.56	14.45
No. 8	Granulocytic	6,000	175	145	155	9.78	12.75
No. 9	Granulocytic	6,500	135	118	165	10.29	11.05
E. S., ♀, 70	Granulocytic	7,300	152	120	145	9.35	10.20
L. H., ♀, 47	Granulocytic	7,000	130	110	130	8.16	11.90
No. 102	Lymphocytic	7,600	125	115	128	17.00	18.70
No. 103	Lymphocytic	7,200	140	150	140	13.69	15.30
No. 104	Lymphocytic	6,300	135	130	140	18.28	17.00
No. 105	Lymphocytic	5,800	145	170	228	32.90	16.15
No. 2803	Lymphocytic	7,700	130	105	100	11.22	10.20
C. A., ♂, 70	Lymphocytic	6,400	135	95	92	11.05	8.50
J. P., ♂, 52	Lymphocytic	5,800	115	128	100	10.20	11.90
J. Q., ♂, 57	Lymphocytic	7,400	140	150	134	10.12	11.05
J. B., ♂, 48	Lymphocytic	6,700	134	160	195	15.30	14.45
Average		6,656.3	133.2	127.6	136.8	13.79	13.39
σ		639.31	17.96	20.73	35.05	5.73	2.827
t		2.67	3.26	3.68	2.8	3.2	1.88

TABLE VIII

Serum protein-bound hexoses in Hodgkin's disease

Case	Type	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
		mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
M. d'A., ♂, 9	Sarcoma	6,700	230	170	190	32.30	34.00
M. F., ♀, 61	Sarcoma	6,200	158	95	158	9.18	15.30
G. M., ♂, 42	Sarcoma	6,500	190	98	100	8.67	14.45
E. G., ♂, 35	Sarcoma	7,700	200	175	204	7.74	13.60
A. M., ♀, 38	Sarcoma	6,700	200	145	210	22.95	15.30
I. diR., ♀, 19	Sarcoma	7,300	221	155	190	15.73	9.35
No. 2811	Granuloma	7,800	92	100	103	11.31	12.75
No. 2930	Granuloma	7,000	215	140	213	11.90	12.75
No. 71	Granuloma	6,800	198	160	175	13.60	11.90
C. M., ♀, 29	Granuloma	6,500	225	182	230	26.35	16.15
A. F., ♀, 61	Granuloma	7,600	153	130	153	5.19	12.75
Average		6,981.8	189.3	141	175	14.99	15.3
σ		516.67	38.92	30.18	43.66	8.201	6.183
t		1.76	5.48	3.65	4.81	2.73	1.72

B. Acute leukemia (Table VI)

There was a statistically significant rise of total hexosamine, total mucoprotein and hexoses bound to protein and mucoprotein fractions.

C. Chronic leukemias (Table VII)

The findings, essentially independent of the histologic type of the disease, were similar to those observed in the acute leukemias, although quantitatively less significant.

D. Hodgkin's disease (Table VIII)

There was a substantial rise in total hexosamine, total mucoprotein and protein-bound hexoses. The rise in mucoprotein-bound hexoses seemed statistically less significant. No appreciable difference was observed between the granuloma and the sarcoma type of the disease.

E. Lymphosarcoma (Table IX)

Total hexosamine, total mucoprotein and mucoprotein-bound hexoses were significantly increased. There was, in fact, a rather spectacular rise in total mucoprotein and mucoprotein-bound hexoses, which seemed typical of the disease. There was no significant difference in findings between patients in this group who had been classified by the pathologist as suffering from either lymphosarcoma or lymphoblastoma.

TABLE IX

Serum protein-bound hexoses in lymphosarcoma

Case	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
R. Z., ♂, 57	6,100	207	150	211	34.27	11.90
No. 36	5,000	190	140	190	51.00	17.85
No. 37	7,500	203	180	301.2	46.75	18.70
No. 38	6,100	135	100	115	10.88	9.35
T. B., ♀, 50	7,700	190	158	200	32.05	11.90
E. M., ♀, 34	7,200	230	170	239	38.93	13.60
S. R., ♀, 38	7,800	255	160	295	33.92	15.30
H. C., ♂, 72	7,000	218	125	179	19.13	11.90
No. 39	7,200	203	120	195	38.68	11.90
T. B., ♀, 54	5,800	290	120	197	29.75	11.90
S. T., ♂, 78	5,600	240	170	279	34.00	13.60
C. M., ♀, 84	7,600	195	140	242	42.50	13.60
E. C., ♂, 43	6,300	280	120	181	31.88	14.45
Average	6,684.6	218.2	142.5	217.2	34.13	13.53
σ	878.67	32.74	23.48	50.42	10.19	2.48
t	1.97	2.33	4.9	6.16	7.07	2.19

TABLE X

Serum protein-bound hexoses in reticulum-cell sarcoma

Case	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
C. B., ♀, 57	6,800	245	170	394.0	38.25	13.60
W. B., ♂, 80	5,600	205	140	160.7	7.74	13.60
J. M., ♂, 51	6,000	210	165	217.0	7.40	16.15
No. 34	7,600	275	130	153.0	32.47	12.75
M. C., ♂, 67	6,500	180	110	195.0	10.80	11.05
Average	6,500	223	143	223.9	19.33	13.43
σ	687.02	33.24	22.27	88.18	13.27	1.648
t	2.62	8.17	4.7	3.62	2.51	2.18

TABLE XI

Serum protein-bound hexoses in mycosis fungoides

Case	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
T. C.	7,600	140	125	273	25.245	11.05
T. H.	7,200	135	110	183	21.505	8.50
P. C.	4,400	135	120	127	24.820	9.35
S. A.	5,600	130	160	137	16.745	6.80
B. B.	6,300	139	204	180	22.270	10.20
Average	6,220	135.8	143.8	180	22.117	9.18
σ	1,146.12	3.54	34.46	51.62	3.044	1.462
t	2.34	4.74	3.28	3.97	9.46	2.2

TABLE XII

Serum protein-bound hexoses in lupus erythematosus disseminatus

Case	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
E. M., ♂, 78	7,200	120	150	195.0	23.375	11.90
H. McC., ♀, 33	6,200	107	135	68.9	6.96	11.05
No. 51	7,400	150	90	190.0	34.00	13.60
Sr. E., ♀, 54	7,600	140	140	123.0	9.18	11.05
S. O'H., ♀, 73	7,100	168.4	155.0	166.0	11.64	12.75
Average	7,100	137.1	134	148.6	17.03	12.07
σ	481.66	21.69	23.108	46.91	10.201	0.9913
t	0.92	2.77	3.68	2.53	2.68	0.93

TABLE XIII

Serum protein-bound hexoses in multiple myeloma

Case	Type	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
		mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
G. G., ♂, 57	Plasmocytic leukemia	10,000	400	170	91	3.57	18.70
No. 21	β-myeloma	9,500	200	160	217	22.27	20.40
N. B., ♀, 62	β-myeloma	10,800	300	180	242	11.15	16.15
J. C., ♂, 59	γ-myeloma	9,100	180	160	117	11.05	17.00
D. S., ♂, 72	γ-myeloma	10,200	200	150	131	19.55	17.00
No. 22	γ-myeloma	9,000	250	170	190	17.00	15.30
Average		9,766.6	255	165	165	14.1	17.43
σ		634.21	76.103	9.574	55.06	6.242	1.68
t		7.6	4.51	12.16	2.81	2.72	5.95

TABLE XIV

Serum protein-bound hexoses in idiopathic thrombocytopenic purpura

Case	Type	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
		mg.%	*mg.%*	*mg.%*	*mg.%*	*mg.%*	*mg.%*
D. H., ♀, 5	Acute	7,600	180	125	92	10.6	12
K. K., ♀, 32	Chronic	6,500	185	160	100	12	15
M. C., ♀, 57	Acute	7,800	218	150	140	11.2	14
I. G., ♀, 56	Chronic	7,200	145	130	125	9.6	16
M. R., ♀, 28	Chronic	7,500	175	150	145	8.9	15
Average		7,320	180.6	143	120.4	10.46	14.4
σ		453.43	23.31	13.27	21.13	1.105	1.357
t		0.07	6.79	6.83	2.29	3.61	3.39

F. Reticulum-cell sarcoma (Table X)

Patients included in this group showed considerable variations in histologic characteristics and clinical course, but equal variability of chemical findings. The statistical analysis revealed significant rise in total hexosamine and protein-bound hexoses only.

G. Mycosis fungoides (Table XI)

There was a significant rise in hexoses bound to both the protein and the mucoprotein fraction, although the increase in total mucoprotein was equivocal. The rise in total hexosamine indicated by the value of σ was apparently of no real significance according to the evaluation of the t test.

H. Lupus erythematosus (Table XII)

There was a superficial resemblance of the findings to those in mycosis fungoides. Statistically, however, the only significant variation was an equivocal rise of the total hexosamine.

I. Multiple myeloma (Table XIII)

Serum of these patients showed a typical pattern. There was a significant rise in total protein, protein-bound hexoses, total and mucoprotein-bound hexosamine.

J. Idiopathic thrombocytopenic purpura (Table XIV)

Total hexosamine and protein-bound hexoses were significantly elevated. The rise in total mucoprotein was equivocal.

K. Disseminated malignancies (Table XV)

Although the sera studied in this group were obtained from a heterogenous group of patients with regard to site of the primary lesion, findings were uniform. There was significant decrease

TABLE XV

Serum protein-bound hexoses in generalized malignancies

Case	Type	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
		mg.%	*mg.%*	*mg.%*	*mg.%*	*mg.%*	*mg.%*
R. R., ♂, 62	Melanosarcoma	6,000	180	130	210.6	11.5	14.0
R. P., ♀, 52	Adenoacanthoma of ovary	6,900	190	135	215	10.2	13.0
H. H., ♀, 60	Adenocarcinoma of breast	6,300	185	130	220	12.5	14.5
J. N., ♂, 60	Carcinoma of prostate	5,900	170	125	177	11.3	13.2
H. H., ♂, 36	Anaplastic carcinoma	5,200	175	138	196	12.2	14.4
H. N., ♂, 55	Carcinoma of rectum	6,700	164	125	173	9.1	13.0
L. S., ♀, 38	Carcinoma of stomach	4,500	167	130	180	10.0	13.8
N. H., ♀, 67	Carcinoma of stomach	7,100	185	142	209	12.7	14.6
Average		6,075	177	131.9	197.6	11.2	13.8
σ		825.76	8.86	5.644	17.48	1.219	0.629
t		4.98	11.94	9.38	14.31	6.84	3.92

in total proteins, with significant elevation of all other serum constituents. Total hexosamine seemed relatively more increased than hexosamine bound to the mucoprotein fraction.

L. Miscellaneous group

Our findings were normal in 25 patients with such conditions as polycythemia vera (four), acquired idiopathic auto-immune hemolytic anemia without evidence of collagen disease or malignancy (three), congenital spherocytosis (four), pernicious anemia (six), iron deficiency anemia (four) and myeloid metaplasia (four).

DISCUSSION

The significance of quantitative changes of the serum mucoprotein fraction in disease has been fully reviewed by Greenspan (12). This author concluded that the total mucoprotein fraction of serum is increased in trauma and in diseases accompanied by inflammatory, neoplastic or degenerative tissue changes, while it is decreased in parenchymal hepatic disease, endocrine dysfunctions and nephrosis. Only fragmentary work, however, seems to have been done on the qualitative composition of the serum mucoprotein fraction in disease. This paper attempts to correlate changes in the mucoprotein fraction of serum in patients with several hematologic diseases as compared with normal sera. This has been done through the study of quantitative variations of some constituents of the serum mucoprotein fraction and of their comparative distribution between the protein and mucoprotein fractions.

Figure 1 and Table XVI are a summary of our work. In Figure 1, the height of columns is based on the calculated averages. With few exceptions, these variations appear significant, as indicated by the results of the t test whose evaluation is offered in Table XVI and is the base for the discussion to follow. The decrease of total protein, noted in most conditions studied on the basis of average figures, was significant only in disseminated malignancies. Absolute rise of serum mucoprotein seemed a common denominator of most conditions studied although significant only in neoplastic processes (acute leukemias, Hodgkin's disease, lymphosarcoma and disseminated malignancies). An increase in protein-bound hexoses was again noted in practically all conditions studied and was statistically significant in the majority of them (acute and chronic leukemias, Hodgkin's disease, reticulum cell sarcomas, mycosis fungoides, multiple myeloma, idiopathic thrombocytopenic purpura and disseminated malignancies). In some conditions (acute and chronic leukemias, mycosis fungoides, idiopathic thrombocytopenic purpura, disseminated malignancies) there was significant increase in mucoprotein-bound hexoses while interesting dissociations were also observed. Thus in Hodgkin's disease and in multiple myeloma only the rise in protein-bound hexoses was significant; in lymphosarcoma, only the rise of mucoprotein-bound hexoses. In mycosis fungoides the rise in mucoprotein-bound hexoses was much more significant than that of the hexoses bound to protein. The opposite situation held true for idiopathic thrombocytopenic purpura. Total hexosamine was also significantly increased in acute and chronic leukemias, Hodgkin's disease, lymphosarcoma, reticulum cell sarcoma, multiple myeloma, idiopathic thrombocytopenic purpura

TABLE XVI

Statistical evaluation of results by the t test: A summary *

	T.P.	T.Ph.	T.Ha.	T.Mp.	Mh.	Mha.
Acute leukemias	−	+	+	+	+	−
Chronic leukemias	±	+	+	±	+	−
Hodgkin's disease	−	+	+	+	−	−
Lymphosarcoma	−	−	+	+	++	−
Reticulum cell sarcoma	−	+	+	−	−	−
Mycosis fungoides	−	+	−	±	++	−
Lupus erythematosus	−	−	±	−	−	−
Multiple myeloma	+	+	++	−	−	+
Idiopathic thrombocytopenic purpura	−	+	+	−	±	−
Generalized malignancies	+(↓)	++	++	++	+	+

* −, insignificant; ±, equivocal; +, significant; and ++, very significant. Variations represented increase from normal with the exception of the group "generalized malignancies," where values for T.P. were lower than normal.

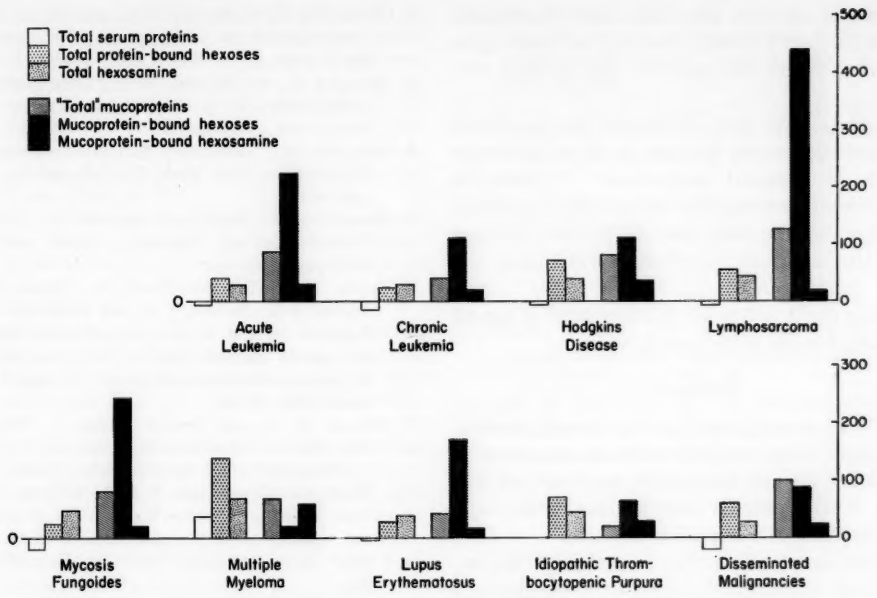

FIG. 1. COMPOSITION OF MUCOPROTEINS IN VARIOUS HEMATOLOGIC DISORDERS—A SUMMARY

Figures represent percentage variations from average normal values, indicated by the horizontal line. Also given for comparison are variations in serum total proteins, total protein-bound hexoses and total hexosamine.

and disseminated malignancies; rise was equivocal in lupus erythematosus. It was of interest that mucoprotein-bound hexosamine was significantly elevated only in multiple myeloma and in disseminated malignancies. In a few instances, the combined variations of the serum constituents produced a distinctive pattern, as in multiple myeloma, lymphosarcoma, and disseminated malignancies. The potential diagnostic value of the changes observed, however, should perhaps be minimized, as these occurred only in patients in fairly advanced stages of the disease.

Also of interest were the findings indicating that the distribution of hexoses between the protein and mucoprotein fractions and the ratio of total hexosamine to mucoprotein-bound hexosamine were much different in normal than in many pathologic conditions. These findings raised the necessary question as to whether the composition of the serum mucoprotein fraction may be different in health as compared to disease and, perhaps, even from one disease state to another. Unpublished electrophoretic studies in this Laboratory have given further support to this possibility, showing an overall anomaly of electrophoretic migration of mucoprotein in disease. Thus, its constituents are usually recovered from the α_1-, α_2-, and β-fractions; in pathologic conditions they may be recovered from the γ-fraction. Other supportive evidence can be obtained from the literature which suggests the largest amount of protein-bound hexoses to be found with the α-globulins in most disease states (13–15). In carcinoma and in rheumatic fever, however, protein-bound hexoses are most abundant in the albumin fraction (16, 17). Bollett (18) has recently confirmed an increase of hexosamine in the mucoprotein fraction of serum in a variety of illnesses, obtaining results which are essentially similar to ours when the same disease states were studied. He found also that such increase was limited to the hexosamine recovered from the α_1 paper electrophoretic fraction. Finally, there is additional evidence, although fragmentary, of a chemical nature. An increase in the ratio hexose: tyrosine in the mucoprotein fraction has been reported in

rheumatoid arthritis, gout (15) and experimental scurvy; it is not found, however, in many other conditions where this ratio has been studied (19, 20).

All these data seem to support the hypothesis that the mucoprotein fraction of serum in disease may be of abnormal composition. It should be remembered, however, that this fraction is of considerable heterogeneity and is possibly nothing more than a mosaic, combining several units. A single increase in content of one of the various fractions might well result in a simulated abnormal structure for the entire mucoprotein.

SUMMARY

1. Total protein, total protein-bound hexoses, total hexosamine, total mucoprotein, mucoprotein-bound hexoses and hexosamine were studied in a series of 111 patients with various hematologic disorders and in 8 healthy individuals.

2. The mucoprotein fraction and total hexosamine were quantitatively increased in diseases of a neoplastic nature. Significant variations of the protein-bound and mucoprotein-bound hexoses were observed in many instances. Fairly distinctive patterns were found in a few diseases (multiple myeloma, lymphosarcoma, and disseminated malignancies).

3. The various anomalies observed suggested that the composition of the mucoprotein fraction of serum may be abnormal in disease.

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